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13. ABSTRACT *(Maximum 200 Words)*

In January 2002 the laboratory of the principal investigator's mentor relocated from The Wistar Institute to the Lankenau Institute for Medical Research (LIMR). Due to this move, as well as the departure of the principal investigator (postdoctoral fellow) from the mentor's research group, transfer of this DoD postdoctoral grant to a new principle investigator is only just now being completed. As a result, limited progress was made during 8 months of the 12 month period covered by this progress report. Aim 1 involving mouse genetics experiments are currently on hold and no new progress is reported. However, in Aim 2, we obtained positive evidence of a role for RhoB and its effector kinase PRK in the antiproliferative action of FTI in rat epithelial and fibroblast cell model systems. Briefly, we demonstrated that RhoB could phenocopy the antiproliferative response to FTI in epithelial cells transformed by activated K-Ras or Rac1 oncogenes, and by using effector mutants of RhoB, we obtained evidence that interactions between RhoB and PRK kinase is involved in this effect. Similarly, manipulating PRK level, localization, or activity in H-Ras-transformed fibroblasts altered the FTI response. This work has been accepted for publication and is currently in press at *Oncogene*.

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Introduction

We proposed to test the hypothesis that RhoB alteration is responsible for mediating FTI action malignant epithelial cells of the breast. Recent studies suggest that prooncogenic Rho proteins play an important role in driving breast cancer, for example, as in highly aggressive inflammatory breast cancers where overexpression of RhoC is a key oncogenic driver [13]. RhoB is an antioncogenic member of the Rho gene family which regulates cellular actin structure, adhesion, motility, proliferation, and survival [6]. RhoB may contribute to the regulation of a signaling cascade that mediates proliferation in response to epidermal growth factor (EGF) [3], a major mitogen for normal and neoplastic breast cells. Moreover, RhoB has been assigned a specialized role in the intracellular trafficking of the EGF receptor [2].

Recent work in our laboratory has identified RhoB as a key target for alteration by farnesyltransferase inhibitors (FTIs), an experimental class of cancer therapeutics that are being tested in clinical trials for breast cancer treatment [9]. In preclinical models, FTIs have displayed relatively unique properties: while largely nontoxic to normal cells they dramatically inhibit the proliferation and/or survival of neoplastically transformed cells. These 'cancer-selective' properties are of significant interest, in part because of they can be traced to molecules other than the molecule that was initially strategized as a target for FTIs, namely Ras [8]. Interestingly, several lines of genetic evidence that have been obtained strongly support a model in which that the antineoplastic properties of FTIs are mediated by a gain-of-function in the antioncogenic RhoB protein [1,5]. Although the main support has derived from mouse models, there is also more recent evidence that RhoB mediates the antineoplastic FTI response in human breast carcinoma cells or RhoC-transformed human mammary epithelial cells [1,10].

The purpose of the presently supported research was focused on two aims. First, we aimed to test whether deletion of the RhoB gene compromised the antitumor response to FTI treatment in mouse models of human breast cancer, as predicted by studies in other model systems. This work was to be performed by breeding RhoB

"knockout" mice to different strains of breast cancer-prone mice, namely the MMTV-neu, MMTV-TGFa, and MMTV-H-ras mice (which are resistant [neu] or susceptible [TGFa, H-Ras] to FTI treatment. Second, we aimed to explore a role in the antineoplastic response for PRK, a key downstream mediator of RhoB signaling in cells. This work was to be performed by determining whether the FTI response mediated by RhoB could be ablated by genetic strategies to suppress or augment PRK signaling in cells. Progress on these Aims is reported.

Body

In January 2002, the laboratory of the principal investigator's mentor relocated from The Wistar Institute to the Lankenau Institute for Medical Research (LIMR). Due to this move, as well as the departure of the principal investigator (postdoctoral fellow) from the mentor's research group who was initially supported by the DoD postdoctoral grant, transfer of the grant to a new principle investigator is only just now being completed. As a result, limited progress was made during 8 months of the 12 month period covered by this progress report. However, of the work that was completed, several reportable outcomes were achieved, in part through collaborative activity with another research group studying the role of RhoB in the FTI response of human breast cells.

Aim 1. This aim included initial mouse breeding experiments to move the rhoB null allele from a rhoB "knockout" mouse onto various 'oncomouse' models for breast cancer. These included the well-documented mice called mouse mammary tumor virus (MMTV)-neu, MMTV-tumor growth factor-alpha (TGFa), and MMTV-H-ras mice. Although matings to generate the desired MMTV*onc*; rhoB-/- strains were initiated, we did not obtain the final strains for testing, due in part to breeding difficulties and unsupported costs related to the relocation of the laboratory. Still, from what breeding experiments and genotyping that was accomplished, we were able to learn that the MMTV-H-Ras strain is unsuited to the study, because of fertility problems in males that appeared to be exacerbated by crossing the rhoB null allele into this strain. MMTV-H-ras mice will not be used for further work. Instead, we will concentrate solely on the

FTI-resistant MMTV-neu strain and the FTI-susceptible MMTV-TGFa strain, neither of which appear to have the same issue as the MMTV-H-ras strain. With regard to Tasks, we partially completed Tasks 1a and 1b during this period.

Aim 2. This aim focused on cell biology experiments to assess a possible role for PRK kinase – a key effector kinase for RhoB signaling - in the FTI response. In initial experiments, we have obtained positive evidence that confirm the hypothesis that RhoB and PRK mediate the suppression of epithelial cell transformation by FTI. We also corroborated this hypothesis by experiments performed in a fibroblast model. Briefly, we demonstrated that RhoB is elevated strongly in cells by FTI, and that ectopic RhoB could phenocopy the antiproliferative response to FTI in epithelial cells transformed by activated K-Ras or Rac1 oncogenes. Using effector mutants of RhoB, we obtained evidence that interactions between RhoB and PRK kinase is involved in this effect. By a second approach, which altered the level, localization, or activity of PRK in H-Ras-transformed fibroblasts, we also obtained evidence in support of the hypothesis that PRK was involved in the FTI response.

The results of the studies described above have been accepted for publication in *Oncogene*, where they are currently in press (see Appendix for manuscript containing experimental details). It should be noted that these studies were conducted in rat intestinal epithelial (RIE) cells, and in established Rat1 fibroblasts, due to arising issues that were related to the use of rodent breast cells in the laboratory. However, we believe the evidence obtained in RIE cells is relevant to breast epithelial cells, based on other work as related below.

With regard to Aim 2 Tasks, we completed Task 2a in the RIE and Rat1 models; fully completed Task 2b; and completed Task 2c in the RIE and Rat1 models. No progress has been made yet on Task 2d; recent preliminary results call into question the rationale behind this task, which may be replaced.

To address questions of the relevance of results obtained in RIE cells, rather than breast cells, we formed a collaboration with the laboratories of Drs. Kenneth van Golen and Sofia Merajver at the University of Michigan, who study malignant transformation of human mammary epithelial cells (HMECs). The studies of Drs. van Golen and Merajver were particularly relevant, because of their focus on RhoC signaling in breast cell transformation and malignant progression [4,11-13], and because of our previous work which suggested that the antioncogenic RhoB protein blocks the prooncogenic RhoA or RhoC proteins in transformed cells [7]. Briefly, the collaborative work showed the following. First, that FTI could elevate RhoB in normal or transformed HMEC. Second, that FTI could dramatically inhibit the transformation of HMEC by RhoC (a model for inflammatory breast cancer [13]), even though FTI could not block RhoC modification (RhoC is resistant to direct inhibition because it is geranylgeranylated in cells, and FTIs do not block protein geranylgeranylation, only protein farnesylation). Third, and most significantly, that ectopic RhoB could phenocopy the antitransforming effects of FTI treatment in RhoC-transformed HMEC. Along with the RIE study, the results supported our hypothesis that elevation of the antioncogenic RhoB protein resulted in inhibition of signaling by the prooncogenic RhoC protein, probably due to competitive interactions with common key effector proteins, such as PRK. The HMEC study performed in collaboration with Drs. van Golen and Merajver was published this year in *Molecular Cancer Therapeutics* (see Appendix for copy of published article containing experimental details and findings).

Key Research Accomplishments

1. Discovery that MMTV-H-Ras mice are unsuitable to Aim 1 studies, due to male fertility problems possibly exacerbated by deletion of the RhoB gene.

2. Evidence obtained in support of Aim 2 studies that the elevation of the antioncogenic RhoB gene is sufficient to mediate suppression of epithelial cell transformation by FTI (in rat intestinal and human breast epithelial cell model systems).

3. Evidence obtained in support of Aim 2 studies that interactions with the RhoB effector kinase PRK are important for the antitransforming effects of FTI in cells.

Reportable Outcomes

1. MMTV-H-ras 'oncomice' are unsuited to studies of the role of RhoB in tumor formation and FTI treatment.
2. FTIs should be considered as a novel treatment for inflammatory breast cancer. In collaboration with Drs. van Golen and Merajver, we showed that RhoB elicited by FTI is sufficient to suppress breast epithelial cell transformation by RhoC. This is an important finding, because it supports the hypothesis that FTI may be useful to treat inflammatory breast cancer – a new idea. Previous to this work, there would be no rationale to use FTI to treat this cancer – RhoC is not directly targeted by FTI action, so FTI would not be expected to be useful against this cancer. However, our studies show that RhoC can be *indirectly* targeted by FTI, through the induction of the antioncogenic RhoB protein that antagonizes RhoC signaling. Therefore, our studies suggest that FTIs should be considered as a possible therapeutic for inflammatory breast cancer.
3. Mechanism of FTI action is connected to PRK interactions. By elevating levels of the antioncogenic RhoB protein, FTIs act to competitively inhibit signaling by prooncogenic Rho proteins that may drive invasion, survival, and metastasis through interactions with key effector kinases such as PRK.

Conclusions

RhoB is further substantiated as a key mediator of FTI action in epithelial cells, including human breast epithelial cells. Elevation of the antioncogenic RhoB protein by FTI may antagonize signaling by RhoC and other prooncogenic Rho proteins, which are frequently overexpressed in breast cancer and causally linked to invasion and metastasis. The mechanism by which RhoB antagonizes transformation may be by competing with prooncogenic Rho proteins (e.g. Rac1, RhoC) for effector molecules, such as PRK.

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Appendices

1. Review articles (2):

Prendergast, G.C. and Rane, N. (2001). Farnesyltransferase inhibitors: mechanisms and applications. Exp. Opin. Investig. Drugs 10: 2105-2116.

Prendergast, G.C. (2001). Actin' up: RhoB in cancer and apoptosis. Nature Rev. Cancer 1: 162-168.

2. Original research articles (2):

van Golen, K.L., Bao, L.W., DiVito, M.M., Wu, Z.F., Prendergast, G.C. and Merajver, S.D. (2002). Reversion of RhoC GTPase-induced inflammatory breast cancer phenotype by treatment with a farnesyl transferase inhibitor. Molec. Cancer. Therapeutics 1: 575-583.

Zeng, P.-Y.*, Rane, N.* , Du, W., Chintapalli, J., and Prendergast, G.C. (2002). Role for RhoB and PRK in the suppression of epithelial cell transformation by farnesyltransferase inhibitors. Oncogene, in press. *co-first authors

Review

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Farnesyltransferase inhibitors: mechanism and applications

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Farnesyltransferase (FT) inhibitors (FTIs) are among the first wave of signal transduction inhibitors to be clinically tested for antitumour properties. FTIs were designed to attack Ras oncoproteins, the function of which depends upon post-translational modification by farnesyl isoprenoid. Extensive preclinical studies have demonstrated that FTIs compromise neoplastic transformation and tumour growth. In preclinical models, FTIs display limited effects on normal cell physiology and in Phase I human trials FTIs have been largely well tolerated. Exactly how FTIs selectively target cancer cells has emerged as an important question, one which has become more pressing with the somewhat disappointing results from initial Phase II efficacy trials. Although FTI development was predicated on Ras inhibition, it has become clear that the drugs' antineoplastic properties are based to a large degree on altering the prenylation and function of proteins other than Ras. One key candidate that has emerged is RhoB, an endosomal protein that has been implicated in selective growth inhibition and apoptosis in neoplastic cells. On the basis of mechanistic studies and other recent developments, we propose that FTIs may be useful to treat a unique spectrum of diseases including not only inflammatory breast cancer and melanoma but also non-neoplastic diseases such as diabetic retinopathy and macular degeneration.

Keywords: angiogenesis, apoptosis, cancer, FTI, Ras, Rho

Expert Opin. Investig. Drugs (2001) 10(12):2105-2116

1. Introduction

Although cancer research has permitted the cure, through surgery and radiotherapy techniques, of many patients with localised cancers, only 10 - 12% of the patients with disseminated cancer are cured by existing treatments [1]. Thus, cancer remains one of the leading causes of death in the developed world. In the past two decades, significant advances have been made in unravelling the molecular mechanisms upon which malignancy is founded. Drug development has followed suit, seeking to identify rational strategies to target cancer cells in a more specific manner than that which has been achieved by empirical approaches [2]. One such strategy has been based upon advances in cancer genetics. It is now known that at least three classes of genes contribute to the pathogenesis of cancer cells, including oncogenes, tumour suppressor genes and genes that ensure faithful chromosomal replication. Mutations in tumour suppressor gene products and DNA repair enzymes typically cause 'loss of function' changes. Proteins suffering 'loss of function' changes offer poor targets for drug development because small synthetic organic molecules are rarely capable of restoring biological activity to mutated proteins. On the other hand, mutations in oncogene proteins cause 'gain of function' changes that offer more attractive targets for drug intervention. The development of FTIs represents an indirect strategy to attack the function of *ras*, which was among the first oncogenes to be defined in human cancer almost twenty years

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ago. As FTI research has recently been considered in significant depth [3-9], this review will focus on the latest advances and issues, with particular emphasis on drug mechanism.

2. *Ras* oncogenes

Ras oncogenes are among the most commonly mutated proto-oncogenes in human solid tumours. Nearly 25% of all human cancers harbour *Ras* mutations that can promote uncontrolled cell proliferation and tumour formation [10]. *Ras* genes have central roles in regulating cell division [11-13]. Their frequent mutation in human cancer has made them a major focus for cancer therapeutic strategies. While three *Ras* genes are transcribed in human cells (H-*Ras*, K-*Ras* and N-*Ras*), the K-*Ras* gene is by far the most commonly mutated *Ras* gene in human cancers. Nevertheless, all mutated *Ras* genes can transform rodent fibroblast and epithelial cell lines and, in co-operation with other oncogenes, can induce transformation of primary cells. Moreover, *in vivo* studies have demonstrated that mutated Ras can drive the formation of spontaneous cancer in transgenic animals [14-16]. Lastly, mutated *Ras* is needed to maintain the malignant status of tumour cells [17,18]. Therefore, drugs that could target *Ras* or *Ras*-induced cell physiology, particularly for K-*Ras*, would be considered good candidates for anticancer interventions.

3. Ras proteins

Ras proteins are small GTP-binding proteins that participate in the regulation of many cellular functions as on-off switches between GTP- and GDP-bound states. The conversion of the inactive Ras-GDP to active Ras-GTP state occurs through interaction with guanine nucleotide exchange factors. In the GTP-bound state, Ras is activated and sends signals to begin cellular processes, including cell growth, differentiation and intracellular signal transduction. These signals are attenuated by interaction with GTPase activating proteins that stimulate GTP hydrolysis and return of Ras to its inactive GDP-bound form. However, mutated Ras lacks GTPase activity and remains stabilised in its active form leading to uncontrolled Ras-driven cell proliferation [12]. In light of this situation, several strategies to block *Ras* functions have been tried:

- restoring GTPase activity to mutated Ras proteins
- inhibiting Ras interactions with downstream effector molecules
- preventing post-translational modification which is required for Ras activity. This strategy has gained the most attention during the past decade [19,20].

The post-translational modification of Ras proteins occurs at their carboxy-terminal tetrapeptide, or CAAX box (where C = cysteine, A = any aliphatic amino acid and X = serine or methionine). The CAAX box serves as the substrate for consecutive enzymatic reactions leading to the formation of mature Ras proteins. The first reaction is isoprenylation at the cysteine residue with farnesyl isoprenoid, an intermediate in cholesterol biosynthesis. The second and third steps are proteolytic cleavage of the three carboxy-terminal AAX residues and the carboxymethylation of the newly isoprenylated cysteine by methyltransferase. Investigations of Ras maturation in cells has spawned major research areas in protein isoprenylation, endoproteolysis and methylation and as many as 0.5% of cellular proteins have been argued to be isoprenylated [21]. Where it occurs, isoprenylation is critical for biological activity and in the case of Ras proteins this even is sufficient for oncogenic activity [22]. Thus, interference with Ras farnesylation has been pursued avidly by many pharmaceutical and academic groups as a strategy to stanch Ras-driven malignancies in the clinic.

4. Farnesyltransferase

Investigations into the maturation of Ras superfamily GTPases led to the discovery of three protein prenyltransferases in eukaryotic cells [23]. Protein prenylation is defined as a covalent modification of thio-ether bonds to C-terminal cysteine residues of cellular proteins. Ras studies identified FT as the enzyme that catalyses Ras farnesylation. In addition, prenyltransferases called geranylgeranyltransferase type-I (GGT-I) and geranylgeranyltransferase Type II (GGT-II) were identified. FT catalyses the transfer of the 15-carbon isoprenyl group on farnesyl diphosphate (FPP) to its protein substrates *via* the formation of a covalent thio-ether bond. Similarly, GGT-I or GGT-II transfers the 20-carbon isoprenyl group on geranylgeranyl diphosphate (GGPP) to its target proteins. FT and GGT-I share a similar mechanism of action for modifying Ras and Rho proteins, while GGT-II is mechanistically different and modifies Rab proteins. All Ras proteins are preferentially farnesylated by FT; however, in the absence of FT activity K-Ras and N-Ras are apparently geranylgeranylated efficiently by GGT-I [24-26]. This 'shunt pathway' for modification of K-Ras or N-Ras has implications for understanding how FT inhibitors inhibit K-Ras or N-Ras transformation, as discussed below.

5. Preclinical studies of FT inhibitors: cell biology and mechanistic questions

As mentioned above, FTIs were developed as a strategy to inhibit Ras-driven tumours, by inhibiting the processing of Ras proteins in cells [19]. There are numerous reviews of the chemical aspects of FTI development in the literature; here we survey biological properties of CAAX peptidomimetic inhibitors that have been most widely investigated. Perhaps the most interesting development in preclinical studies has been the emergence of strong evidence that the antineoplastic properties of FTIs are not so readily traced to Ras inhibition as to alterations in the prenylation status of other proteins in cells [7,27,28].

The FTI response in Ras-transformed cells in monolayer cell culture has several hallmarks, including marked cell

enlargement, flattening and actin stress fibre formation in cell culture [29]. FTIs inhibit both anchorage-independent and anchorage-dependent growth of Ras-transformed cells, with H-Ras-transformed cells exhibiting the greatest sensitivity in terms of morphological changes as well as growth inhibition. Growth inhibition has been reported in various studies with preferential arrests or delays in the G_1 or G_2/M phases of the cell cycle, sometimes with co-ordinate activation of p21WAF1, the cell cycle kinase inhibitor [30-36]. In some human tumour lines, G_2/M arrests have been associated with an alteration of cyclin B1 levels and with effects on spindle pole formation and chromosome alignment [35,37]. Apoptosis is marked in Ras-transformed cells only under conditions where cells are deprived of growth factors or forced into suspension [38-40]. However, apoptosis has been reported in human tumour cell lines in limited cases [35,41].

Pronounced morphological or actin changes do not occur in all transformed cell types where growth inhibition may be apparent. It is notable, however, that such shape and actin fibre changes also occur in normal cells, where growth inhibition is fairly negligible [29]. In these cells, the loss of anchorage-independent growth correlates with reversion to a flat cell phenotype with division rates similar to normal cells [29]. Cells transformed by non-Ras oncoproteins such as v-Raf that do not undergo prenylation are not affected by FTIs, consistent with specificity towards the post-translational modification event on Ras [42]. However, the presence of mutant Ras is not a prerequisite for response: for example, one study of human cancer cell lines showed that more than 70% of cells were sensitive when treated with low micromolar concentrations of FTI, but that cells harbouring mutant Ras were not necessarily any more susceptible to growth inhibition than cells harbouring wild type Ras [43]. A caveat to these observations was provided by evidence of a correlation between the FTI response and Ras-GTP status (rather than mutant Ras status) in a study of astrocytoma cell lines [44]. Nevertheless, it is striking that FTIs can inhibit malignant growth induced by activated K-ras or N-ras, despite the fact that under such circumstances these proteins are geranylgeranylated by GGT-I and in principle remain active [16,24-26,45,46]. In a combinatorial study, FTIs were sufficient to achieve the same extent of growth inhibition in K-Ras-transformed cells whether or not GGT-I inhibitors were included [47]. FTIs can also inhibit cell transformation by H-Ras proteins that are engineered to be modified by N-myristoylation or geranylgeranylation [48,49]. There is consequently considerable evidence that FTIs can control cancer cell growth in a Ras-independent fashion, as we have argued elsewhere [50].

6. Preclinical studies of FT inhibitors: tumour biology

A wide variety of FTIs have been tested for antitumour activity in tumour xenograft assays performed in nude mice. In these studies, tumours that were tested for drug response were derived either from oncogene-transformed rodent fibroblasts or human tumour cell lines. The results of such studies have been surveyed elsewhere recently [4,9,51]. Recent reports and some key studies are summarised below.

The FTI R115777 showed cell growth inhibition in 75% of the 53 human tumour cell lines tested, independent of *Ras*-mutational status [52]. when administered orally to nude mice bearing mutant H-*Ras*, mutant K-*Ras* and wild type *Ras* genes, R115777 showed significant antitumour effects. However, tumour cell lines bearing mutated K-*Ras* genes required high doses [52]. In another study, SCH-66336 showed very specific antitumour activity against BCR/ABL leukaemic cells as well as human astrocytomas cell lines [44,53]. SCH-66336 also inhibited primary human cells derived from chronic myeloid leukaemia [54]. Interestingly, mice treated with SCH-66336 for BCR/ABL-induced leukaemia remained disease-free for more than a year [54]. A G_2/M arrest was noted in this model. In another study, BIM-46228 reduced the growth rate of xenografted human tumour cell lines with mutated K-*Ras* gene but no tumour regressions were observed [55]. Also, an *in vitro* combined therapy of FTI with chemotherapeutic agents and radiotherapy proved to be beneficial as a combinatorial regimen [55]. Experiments in transgenic 'oncomouse' models have previously demonstrated the antitumour efficacy of FTIs. In the H-*Ras* 'oncomouse' model, where FTIs display robust tumour regression activity in the absence of apparent toxicities [56], FTI L-744,832 was shown to elicit regression of large mammary tumours elicited by H-Ras or Myc/H-Ras induction in manner associated with p53-independent cell cycle inhibition and apoptosis [31]. Regression associated with apoptotic induction was also observed with the FTI SCH-66336 in the related WAP/H-Ras 'oncomouse' model, which is also subject to mammary tumours [57].

Several groups have reported additive or synergistic effects of FTI treatment with classical chemotherapeutics. FTI SCH-66336 potentiates the effects of paclitaxels against human tumour xenografts [58], consistent with the results of a similar study of FTI-2148 [59] and with *in vitro* studies of FTI L-744,832 against human tumour cell lines [60]. FTI SCH-66336 was also reported to synergize in a sequence- and cell line-specific manner when added with cisplatin to several human tumour cell lines [61]. While this study did not test the combination in mice, its findings were consistent with previous *in vitro* studies of L-744,832 that evidenced co-operation with gamma irradiation [62,63], as well as with an earlier report that FTI-2148 can synergize with DNA damaging agents to block tumour growth in mice [59]. The latter study noted collaboration not only with paclitaxel and cisplatin but also gemcitabine [59], the latter of which has shown some promising efficacy in combination with FTIs in human trials (see below).

7. Clinical trials

A summary of the results from initial Phase I and Phase II clinical testing of several different FTIs has recently been considered in depth elsewhere [8,51]. To date, several FTIs have been evaluated in such trials as a single agent or as part of a combinatorial regimen with other cancer chemotherapeutics. FTIs including L-778123, R115777, SCH-66336, BMS-214662, CP-609754 and AZD-3409 are being assessed for antitumour activities with less toxicities. One of these molecules, R115777, has entered Phase III testing for pancreatic and colorectal cancer.

L-778123 was evaluated in a continuous 14- or 28-day iv. at 140 - 560 mg/m^2/day schedule. The dose limiting toxicities were QTc prolongation, grade 4 neutropenia, fatigue and myelosuppression. A combination trial with radiotherapy of a total of 21 patients resulted in 2 complete and 4 partial responses with dose levels between 280 - 560 mg/m^2/day for 1, 2, 4, 5 and 7 weeks schedule. A general grade III diarrhoea and grade IV haematological toxicity was seen. A dosage of 280 mg/m^2/day is recommended for Phase II trials for a number of solid tumours, including those of the pancreas, breast, colon and lung.

R115777 has been evaluated for relapsed and refractory acute leukaemias. An oral b.i.d. dosage of 100, 300 , 600 and 900 mg for 21 days for up to 4 cycles were administered. Four of 20 patients had stable disease during treatment, two other patients showed normal reconstitution of haematopoiesis with out any toxicities. In another study, administration of R115777 at a dose of 50 - 500 mg b.i.d. showed partial remission in one patient with dose limiting toxicities at 400 mg, which included neutropenia, thrombocytopenia and fever. A 300 mg b.i.d was considered as a future chronic dosing level for Phase II trials. During Phase II trial, three out of 26 breast cancer patients showed partial response, while 9 other patients showed stable disease at the 3 months evaluation time. Currently, this drug has entered into Phase III trials for wide variety of solid tumours. A combination regimen with either gemcitabine or capecitabine showed well tolerated dosage with less toxicity levels.

Recently, a study involving oral administration of SCH-66336 was completed in patients with advanced solid tumours. These patients were given different dose levels of FTI up to 400 mg, twice daily. A dose-limiting toxicity at 400 mg consisted of gastrointestinal toxicities along with neutropenia and thrombocytopenia. However, at 200 mg SCH-66336 was safely administered on long-term protocol without any myelosuppression toxicity [64]. A combinatorial therapy with paclitaxel and gemcitabine was also evaluated in a Phase I trial for solid tumours. Out of 18 evaluable patients, 6 patients showed partial responses with minor toxicities for paclitaxel, whereas 2 partial and 2 minor responses were achieved with gemcitabine treatment. In addition, 11 of 25 patients showed stability of disease for > 6 months.

BMS-214662 has entered Phase II trials for pancreatic, head and neck, lung and colorectal cancers. In the Phase I trial BMS-214662 was given as a 1 h infusion with a starting dose of 56 mg/m^2 through 209 mg/m^2, ranging from 1 - 30 weeks of treatment. Toxicity ranged from grade 3 neutropenia, vomiting and hypotension at the higher doses.

Several other compounds, including CP-609754 and AZD-3409, have also entered into clinical trials but as yet no data is available from these studies.

In summary, some might judge the results of Phase II testing of FTIs to be rather disappointing, based on the promising preclinical data which had accumulated previous to human trials. However, combinatorial applications of FTIs with other cancer chemotherapeutics shows sufficient promise at this juncture to be worth pursuing.

8. Mechanisms of action: evidence that RhoB is a critical target of FTIs

FTIs were designed as a strategy to inhibit Ras. However, perhaps the most pressing question in the field is whether the antineoplastic effects of FTIs are actually related to Ras inhibition. There are few doubts that Ras may be a sufficient target but is it necessary? The doubts that inhibition of mutant Ras is indeed crucial for FTI action have previously been discussed in detail [4,6,27,28,50,51]. Some of the earliest studies of FTI action raised anomalies in the model that FTI action was based directly in Ras inhibition, even in the case of H-Ras which cannot be geranylgeranylated in FTI-treated cells like K-Ras or N-Ras. First, the kinetics of morphological reversion observed in H-Ras transformed Rat1 cells upon FTI treatment were too rapid to be explained by depletion of processed H-Ras from cells [29]. Given that the half-life of fully modified H-Ras is 24 h, processed H-Ras cannot be depleted significantly from cells for 2 - 3 days after FTI treatment (since FTIs block the H-Ras prenylation rather than the steady-state level or activity of mature H-Ras). However, reversion of H-Ras transformation is achieved within 18 - 24 h of drug treatment, when modified Ras persist at ≥ 50% of their pre-treatment level. In addition, once cells are treated with FTIs, the reverted phenotype persists for several days after the FT activity and fully modified Ras returns to pre-treatment levels [29]. This interesting long-lived phenotype has been traced to upregulation of collagen Iα2, a long-lived growth inhibitory collagen isoform that Ras must suppress in order to transform fibroblasts [65,66] and that FTIs must derepress to induce and maintain the reverted phenotype [67]. Thus, studies in H-Ras-transformed cells suggested that neither initiation nor maintenance of reversion to a non-transformed phenotype by FTIs was well correlated with steady-state depletion of farnesylated Ras proteins. A converse set of observations indicates that FTIs can inhibit transformation by Ras proteins that are resistant to direct inhibition by the drugs, including N-myristylated and geranylgeranylated Ras isoforms, K-Ras and N-Ras, as mentioned above. Moreover, there are explicit examples where

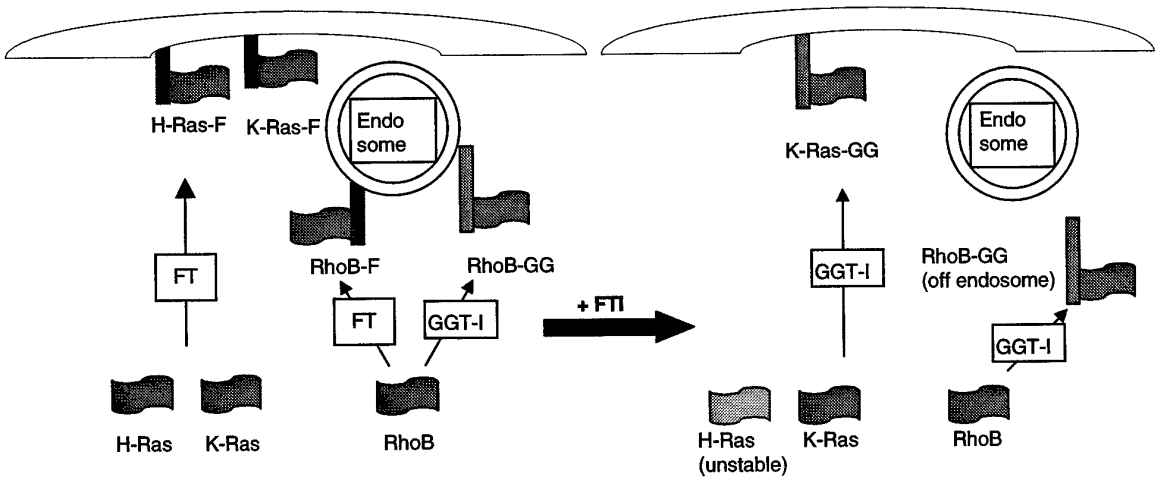

Figure 1. How FTIs affect RhoB *versus* Ras proteins. Newly synthesised precursor proteins are normally modified by farnesyl transferase (FT) or geranylgeranyl transferase (GGT-I). FT inhibitors (FTIs) block the farnesylation of H-Ras, resulting in a loss of function. In contrast, K-Ras becomes geranylgeranylated by GGT-I in FTI-treated cells, remaining membrane-associated and active. FTI also blocks synthesis of RhoB-F, but RhoB-GG accumulates due to geranylgeranylation of all newly synthesised protein by GGT-I. The shift in prenylation pattern of RhoB is associated with elevated expression and mislocalisation of RhoB-GG away from its normal endosomal location in cells.

blocking H-Ras farnesylation is insufficient to block H-Ras transformation itself [49,68]. More extreme sceptics might even entertain doubts that the antineoplastic effects of FTIs are related to FT inhibition, as the biology of FTIs has not yet been explored in cells that lack FT itself (e.g., in mouse cells where the gene encoding the β subunit of FT has been homozygously deleted). However, the correlations reported for most FTIs are sufficiently tight to reasonably argue against this extreme view, at least in most cases. In contrast, given the many clear indications in the literature, many investigators have come to the view that many biological properties of FTIs may not be based solely upon Ras-dependent mechanisms.

What other potential drug targets have been identified and what causal relationships have been developed? A leading candidate is RhoB, a small GTPase in the Ras superfamily which can be both farnesylated and geranylgeranylated in cells [69,70]. RhoB is a member of the Rho family of small GTPases involved in regulation of the actin cytoskeleton, vesicle trafficking and numerous other cell processes. Two recent reviews muster the compelling evidence that RhoB alteration is a cause of the cellular response to FTIs [7,71]. Interestingly, RhoB responds to FTI treatment by a gain-of-function alteration, not a loss-of-function alteration as in the case of H-Ras. The gain-of-function alteration is critical to mediate actin-based phenotypic changes, growth inhibition and apoptosis in FTI-treated cells. The initial clue for the involvement of a Rho protein in the FTI response was the observation that FTIs can induce actin stress fibres in normal cells [29]. RhoB responds rapidly to FTI treatment, with kinetics that are consistent with biological response as mature RhoB proteins turnover quickly [49].

RhoB is a direct target of FT in cells but it responds in a complex manner to FTI treatment because of its dual prenylation status in cells. FTIs block production of the farnesylated RhoB isoform (RhoB-F) but cause an elevation of the geranylgeranylated RhoB isoform (RhoB-GG), due to the continued activity of GGT-I in FTI-treated cells [70]. This shift in prenylation pattern is correlated not only with an elevation in the level of RhoB-GG but also a change in its intracellular localisation [49]. A contributing factor in the elevation of RhoB-GG in FTI-treated cells appears to be increased transcription of the *rhoB* gene (S Sebti, personal communication), an effect probably related to the fact that the gene is autoregulated [72]. Thus, FTIs alter RhoB in a radically different manner than Ras (Figure 1).

FTIs elicit a loss of RhoB-F and a gain of RhoB-GG. It has become apparent recently that the gain of RhoB-GG is a crucial event, since this is sufficient to phenocopy the FTI response [33,34]. The RhoB-GG isoform used to demonstrate this effect was engineered by substituting the normal C-terminus of RhoB with the C-terminus of RhoA, which can only direct geranylgeranylation [69]. Expression of this engineered isoform, which mislocalizes like wild type RhoB in FTI-treated cells [69], is sufficient to mediate growth inhibition and apoptosis in human tumour cells as well as in transformed rodent cells [33,34]. In contrast, RhoB-GG had little effect on untransformed rodent cells and is therefore similar to FTI treatment [33]. RhoB-GG is crucial for apoptosis and actin reorganisation induced by FTIs, in particular, as demonstrated by the defective drug response of cells that are nullizygous for the *rhoB* gene [68]. It should be noted that RhoB-

Table 1. Summary of FTI trials in human subjects.

Drug	Trials	Tumours	Route	Schedule	MTD	Responses
L-778123	With radiotherapy	Solid	iv.	1, 2, 4 and 5 weeks	280 - 560 mg/m^2/ day	2 CR, 4 PR Grade III diarrhoea and grade IV haematological toxicities
	Phase II (not evaluated)	Solid				
R115777	Monotherapy/ Phase I	Acute leukaemias	Oral	Chronic b.i.d for 21 days	300 mg b.i.d	6 PR, no significant haemotoxicities
		Solid		Chronic b.i.d	400 mg b.i.d	Neutropenia, thrombocytopenia, fever
	With capecitabine	Solid	Oral	14 days every 3 weeks	300 mg b.i.d	1 MR, 1 stable
	Monotherapy/ Phase II	Advanced breast	Oral		400 mg b.i.d	3 PR, 9 stable, myelosuppression
SCH-66336	Monotherapy/ Phase I	Solid	Oral	1 week; followed by 3 weeks rest	25 - 400 mg b.i.d	Well tolerated
	With paclitaxel	Solid	Oral	With 4 different dose levels	100 - 150 mg b.i.d with 135 - 175 mg/ m^2 paclitaxel	6 PR
	With gemcitabine	Solid	Oral	1 week	150 mg with 1000 mg/m^2 of gemcitabine	2 PR, 2 MR, 11 stable
	Phase II (not evaluated)	Solid				
BMS- 214662	Monotherapy/ Phase I	Solid	iv./oral	1 h infusion, with either iv. or oral every 3 weeks	36 - 225 mg/m^2	1 MR with gastrointestinal toxicity
	Phase I	Solid	iv.	1 h infusion for 1 - 30 weeks	56 -209 mg/m^2	1 MR
CP-609754	Phase I (not evaluated)	Solid				
AZD-3409	Phase I (not evaluated)	Solid				

CR: Complete response, MR:Minor response, PR: Partial response.

GG can selectively inhibit the growth of FTI-susceptible but not FTI-resistant human carcinoma cells [34]. Thus, RhoB alteration is applicable to the FTI response in both rodent model systems as well as human epithelial cancer cells.

As an FTI target, RhoB can address the long-standing puzzle of why FTIs have so few effects on the growth or differentiation of normal cells. Recently, gene targeting experiments in mice have demonstrated that the *RhoB* gene can be homozygously deleted without affecting development, fertility or wound healing [73]. Thus, it can be inferred that *any* effect of FTIs on RhoB function would be inconsequential to normal cells, since RhoB can be eliminated altogether without discernible effect. The dispensability of RhoB to normal cell physiology in mice is interesting in light of the lack of apparent toxicity of FTIs to normal cells in mice [56]. Like FTIs, elevated levels of RhoB-GG do not affect the growth of normal cells even though this event is sufficient to dramatically affect the physiology of FTI-susceptible neoplastic cells [33]. Therefore, it is apparent that RhoB has a peculiar transformation-associated role that mirrors the properties of the FTI response. Although the physiological functions of RhoB remain to be determined exactly, on the basis of 'gene knockout' studies in the mouse it would appear that RhoB plays a role as a negative modifier gene in stress-associated processes, including cancer [73]. The findings of this study, which support a negative-acting role for RhoB in cancer cells, are consistent with the evidence that the antineoplastic effects of FTIs are mediated by a gain-of-

function in RhoB. In conclusion, a self-consistent picture emerges from the FTI investigations and the mouse genetic analysis, supporting the notion of RhoB as a key FTI target, which acts through gain-of-function.

9. RhoB and apoptosis in cancer cells

In support of its role in mediating apoptosis by FTIs, RhoB was recently demonstrated to be crucial for the apoptosis induced in transformed cells by DNA damaging agents or paclitaxel [74]. This requirement was defined using the 'gene knockout' mouse embryo fibroblast (MEF) model system that was used to define the requirement for RhoB in FTI-induced apoptosis [68]. As in that case, loss of RhoB did not compromise growth arrest but stanched apoptosis after cellular exposure to gamma irradiation, doxorubicin, or paclitaxel. This study also established that RhoB was crucial for the previously described ability of FTIs to co-operate with DNA damaging agents [74]. In the absence of the *rhoB* gene, FTIs could not elicit RhoB-GG and could not sensitise cells to gamma irradiation or doxorubicin, thus further linking RhoB alteration to the FTI response in transformed cells. In summary, RhoB appears to be a crucial player in the apoptotic response of cells, not only to FTIs but also to DNA damaging agents and antimicrotubule agents. The latter two agents represent major classes of therapeutics in the arsenal of the clinical oncologist.

10. RhoB as a target for prenyltransferase inhibitors

A recent study demonstrating that RhoB overexpression is growth inhibitory in tumour cells, whether expressed as wild type, farnesylated or geranylgeranylated isoforms, has led one group to argue that RhoB cannot be a key FTI target [75]. The arguments used to arrive at this conclusion can be shown to be invalid. However, the results of the study prompt the interesting idea that RhoB may be a key target for GGT-I inhibitors as well as for FTIs. The argument raised against RhoB as an FTI target derive from the question as to whether the functions of RhoB-F and RhoB-GG differ or not. There is some support for distinct functions ([70] and G Favre, personal communication) but also for overlapping functions [34,75]. The possibility of overlapping functions does not weaken the argument that RhoB is a primary FTI target and to argue in this manner is invalid. FTI depletes RhoB-F from cells, so any inhibitory effects of RhoB-F elevation are irrelevant to the FTI response. Similarly, the argument that the loss of RhoB-F is not required for the FTI response is an invalid argument (termed a 'straw man' argument in logic), as it is not loss of RhoB-F that is important but rather gain of RhoB-GG. The former event is only important insofar as it is a prerequisite for the RhoB-GG elevation and mislocation needed to elicit the drug response. This smaller role for RhoB-F loss is still important, however, because without it the F:GG imbalance that is needed for RhoB-GG to accumulate and mislocalise would not occur. Thus, RhoB-F has a staging role rather than a functional role in the FTI response and only RhoB-GG is crucial. As discussed in more detail below, the bottom line is that FTIs may use RhoB (in the guise of RhoB-GG) to interfere with certain Rho signals in cells.

Could GGT-I inhibitors also use RhoB, in this case RhoB-F, to mediate growth inhibition? In contrast to FTIs, GGT-I inhibitors increase the relative level of RhoB-F instead of RhoB-GG in cells. It is in this context that the growth inhibitory effects of RhoB-F are relevant and potentially interesting. With this expanded scope in mind, RhoB can be invoked as a generalised target of FTIs or GGT inhibitors. In short, the inhibitory effects of RhoB-F may be sufficient for growth inhibition by GGT-I inhibitors, in the same way that RhoB-GG is sufficient for growth inhibition by FTIs. Each class of inhibitor would use RhoB (in the guise of either RhoB-F or RhoB-GG) to interfere with cell growth. What isn't known yet is whether changes in the ratio of F:GG isoforms elicited by each class of prenyltransferase inhibitor will affect RhoB function by directly affecting its localisation. In any case, it is important that FTIs not only elevate RhoB-GG but also mislocalise it [49]. Since localisation is probably compromised to some degree by overexpression, it will be important to test the response of cells and animals lacking RhoB to GGT-I inhibitors, as was done for FTIs, to obtain genetic proofs for or against the relevance of RhoB alteration to GGT-I inhibitor responses in cells.

11. Expert opinion

11.1 RhoB action

Unravelling the mechanism by which FTIs act is important for at least two reasons. First, mechanistic insight may promote more effective application of FTIs against disease, by allowing appropriate Phase II trials to be designed. Second, mechanistic insight may lead to a better understanding of neoplastic pathophysiology or to the problems that lie at the roots of cancer. Therefore, how does RhoB-GG act? Recent findings suggest that it may counter or interfere with Rho signals needed for the proliferation and perhaps the viability of transformed cells. Rho proteins regulate a wide spectrum of cellular processes. Members of this family, including the prototypes RhoA, Rac1 and Cdc42, were initially identified as regulators of cytoskeletal actin organisation. However, it has become clear that Rho proteins have many other roles in cells, include the regulation of adhesion, motility, vesicle trafficking, cell cycle progression, cytokinesis and transcription. This diversity of roles is reflected in the large number of effector signalling pathways activated by various Rho proteins, as well as the intimate linkages between them. There is increasing evidence that Rho proteins influence a variety of important processes in cancer, including cell transformation, survival, invasion, metastasis and angiogenesis. Although Rho proteins do not

appear to be mutated in cancer cells, their expression is often elevated, suggesting that Rho dysregulation promotes malignant phenotypes. There is increasing interest in the key role that Rho proteins may play in driving the complex phenomena that underlie tumourigenesis.

As summarised above, RhoB can mediate growth inhibition and apoptosis in neoplastic cells exposed to FTIs or apoptosis of such cells exposed to DNA damaging agents or paclitaxel. These roles contrast with other Rho proteins, such as RhoC, which have positive roles in cancer [76-79]. The effector domains of RhoA, RhoB and RhoC are virtually identical and in several cases 'RhoA effectors' have been confirmed to interact with RhoB. Thus, moving RhoB between different cellular compartments, as FTIs appear to do, may allow RhoB to compete for effector functions that are normally unavailable. Competition for Rho GEFs, GAPs or GDIs is conceivable, widening the scope of RhoB action to include many Rho, Rac or Cdc42 proteins, which share many of these factors. In this way, RhoB could act as a 'natural' dominant inhibitor that disrupts or counters Rho signals involved in proliferation, survival, or transformation (RhoB-GG effects elicited by FTIs may represent one mechanism for how this can be done).

The concept of RhoB as a natural inhibitor of transforming Rho signals is supported by recent work. Ectopic expression of RhoB-GG can phenocopy the ability of FTI L-744,832 to effectively inhibit the anchorage-independent growth of K-Ras- or Rac-transformed rat intestinal epithelial cells (RIE cells) (PY Zeng, W Du and GC Prendergast, unpublished observations). Similarly, ectopic expression of RhoB-GG can phenocopy the ability of FTI L-744,832 to effectively inhibit RhoC-dependent transformation of human mammary epithelial cells (HMECs) (K van Golen and S Merajver, manuscript in preparation). Since FTI L-744,832 can not directly block the prenylation of K-Ras, Rac1, or RhoC (the latter of which are normally geranylgeranylated in cells), the drug must act by some indirect route such as by eliciting RhoB-GG. The ability of RhoB-GG to mimic the drug effect in consistent with previous findings in H-Ras-transformed Rat 1 fibroblast models [33]. The simplest interpretation is that FTIs act by eliciting RhoB-GG and that RhoB-GG acts as a 'dominant inhibitor' of Rac1 or RhoC signals required to maintain the transformed phenotype, in either rodent or human epithelial cells. The fact that FTIs cause a mislocalisation as well as an elevation of RhoB-GG fits well with the classical genetic notion of a dominant inhibitor. A further prediction of this model is that genotoxic stresses, which apparently require RhoB to activate apoptosis, should both elevate and alter the localisation of one or both RhoB isoforms. The first prediction is supported by a report that the *RhoB* gene is induced by genotoxic stress [80]. Exactly how RhoB-GG may influence apoptotic susceptibility is not yet clear, although biochemical linkages between RhoB and Akt, NFκB, or G_2/M events *via* the Rho effector mDia are being entertained ([41,71,81,82] and L Benjamin, personal communication).

11.2 Where the RhoB mechanism leads: cancer applications

The hypothesis that RhoB counters pathological Rho signals leads one to consider the use of FTIs against a wider spectrum of diseases than originally planned. With regard to cancer, one may wish to apply FTIs to counter types of malignancy where Rho signals are oncogenic drivers. The roles for positive-acting Rho and Rac proteins in cancer is an exciting area that has emerged recently [76,77,79,83-86]. However, at the current time there is less information about what settings Rho proteins might be critical drivers of malignancy. However, recent evidence suggests that RhoC may be such a driver in inflammatory breast cancer and in malignant melanoma [78,79]. Since RhoB-GG is sufficient to phenocopy the effects of FTIs in RhoC-dependent transformation (as mentioned above), one might propose that FTIs could be useful agents to treat inflammatory breast cancer and melanoma. Both these tumours tend to lack Ras mutations and so would not have received attention for clinical testing with FTIs. A Phase II trial may begin based on the mechanistic advance implicating RhoB in the FTI mechanism as well as the intriguing preclinial data mentioned [87].

11.3 Where the RhoB mechanism leads: other applications

FTIs may be useful in neoplastic but also non-neoplastic diseases that are marked by pathological angiogenesis. Despite the significant interest in anti-angiogenic strategies for cancer therapy and intensive investigation of FTIs, these two research areas have not overlapped significantly to date. This should change shortly given evidence that FTIs have anti-angiogenic activity that in some settings may be RhoB-dependent. Several years ago, Bob Kerbel and his colleagues demonstrated that FTIs could inhibit H-Ras-induced elevation of vascular endothelial growth factor (VEGF) and they suggested that this might partially explain the tumour-selective effects of FTIs in animals [88]. In H-Ras-transformed Rat1 fibroblasts, VEGF suppression by FTIs is mediated at the level of RNA accumulation, including mechanisms that affect RNA turnover and/or transcription (W Du, unpublished observations). Although this work was not followed up, another group subsequently described the anti-angiogenic effects of FTIs tested in a set of *in vitro* and *in vivo* models [89]. This line of investigation has recently been extended significantly in Laura Benjamin's laboratory, which has demonstrated that FTI L-744,832 exquisitely inhibits sprouting angiogenesis during retinal development. Notably, non-sprouting angiogenesis is unaffected in mice treated with FTI, in contrast to VEGF inhibitors, which broadly inhibit both types of angiogenesis in animals. Interestingly, FTI effects are associated with rapid apoptosis of sprouting endothelial cells in the retina, are correlated with Akt suppression and exhibit a requirement for RhoB (I Adini, N Sater, AX Liu, GC Prendergast and LE Benjamin, manuscript submitted). The latter finding suggests that the ability of FTIs to elicit RhoB-GG is important for anti-angiogenesis activity. Based on the discus-

sion above, one might rationalise the observations based on a requirement for Rho signalling in the survival, motility and/or polarity of sprouting endothelial cells.

These observations are interesting because they suggest that FTIs might be useful to treat pathological but non-neoplastic forms of angiogenesis that characterise certain diseases, such as diabetic retinopathy and macular degeneration. These diseases are widespread among the elderly and cause blindness. They are treatable by laser therapy in the opthalomology clinic, but to date there is not an orally available small molecule therapy, as might be provided by FTI, which would consequently displace this current treatment regimen. A Phase II trial to test the efficacy of FTI in such disorders might be initiated based on the intriguing mouse data presented above. As in cancer settings, a link to RhoB alteration is appealing, given the apparent function of RhoB in stress environments that do not characterise normal cells.

11.4 Conclusion and expert opinion

The above discussion aims at illustrating the potential utility in determining how FTIs act in cells. Although originally developed as an anti-Ras agents, FTIs have been shown to act through non-Ras targets to a rather large and unexpected extent. The utility of mouse genetic analysis lends sound support to the case for RhoB as a key target. A shift from Ras to Rho in thinking about how FTIs elicit various biological responses leads one to new vantages on how these drugs might be exploited in the clinic. Phase I trials have determined efficacious dosages with minimum toxicity as regards cancer. Additional Phase I trials may be required to assess the non-neoplastic applications for FTI suggested here. In Phase II trials, FTIs need to be further evaluated for tumour types and thus appropriate patient populations. Given evidence of role for RhoB in the drug response and the evidence that RhoB alterations can interfere with Rho transforming signals, tumours driven by Rho, such as inflammatory breast cancer or melanoma, may be a good choices for clinical evaluation. Recent work suggests that FTIs should also be tested to treat non-neoplastic diseases marked by pathological angiogenesis, such diabetic retinopathy or macular degeneration. Extending the RhoB paradigm may also suggest applications in other diseases characterised by hyperplastic pathology. FTI research would be anticipated to continue its move in the direction of mechanistic insights and its subsequent translation into clinical applications.

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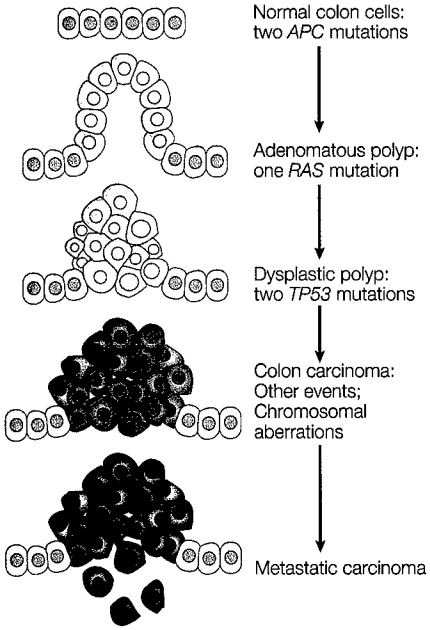

Figure 5 | **A possible five-hit scenario for colorectal cancer, showing the mutational events that correlate with each step in the adenoma–carcinoma sequence.** Based on a model from Fearon and Vogelstein (REF. 47).

birth rate and decreasing death rate. Interestingly, loss of *TP53* leads to defective centrosome replication and numerous chromosomal abnormalities[46], the feature of cancer that first attracted the notice of von Hansemann and Boveri.

Inactivation of several other cloned tumour-suppressor genes, including the *APC* gene of FAP, is associated with hereditary cancer and with benign precursors of malignant tumours. These benign lesions, usually adenomatous, are all 'two-hit' tumours that are found in large numbers in the target tissues, undergo malignant transformation at low frequency and require other mutations to do so. These genes, including *APC*, seem to inhibit passage through the cell cycle, so their loss or inactivation increases cell birth rate. In many cases, the transition to frank malignancy involves loss or inactivation of *TP53* (REFS 47,48), thereby reducing cell death rate. Mutations and losses of these two genes could account for four events in the pathway to colon cancer.

The well-known 'adenoma–carcinoma' sequence in colorectal cancer has made this disease a popular model for a multihit cancer[47]. Events on the path to cancer include not only mutations in *APC* and *TP53*, but also in one copy of the *Ras* oncogene[47]. This path would seem to involve five mutational events, a number that is quite compatible with David Ashley's estimate of four or five, which, as described earlier, was calculated from a comparison of log–log plots of age-specific colon cancer incidence in normal and FAP persons, long before we knew of the existence of oncogenes or tumour-suppressor genes (FIG. 5). This number of events could occur at normal spontaneous mutation rates — given the number of cell divisions that occur in the colon over many years, and the clonal expansion that occurs because of selection for mutants that have increased growth rates and decreased death rates from apoptosis during cell turnover[49]. However, the transition from polyp to carcinoma has been reported to be associated with occult genomic instability[50,51], as judged by changes in DNA that are not associated with visible karyotypic abnormalities. It seems that DNA lesions are normally repaired by processes, such as recombinational repair, that leave the chromosome intact. When the induction of this repair is compromised, apoptosis should ensue. This process fails in the presence of *TP53* mutations, and florid karyotypic changes emerge abruptly. This is the state of chromosomal instability (CIN)[52].

Although it is true that some cancers show only one or a few chromosomal abnormalities, most are, like colon cancer, very abnormal at diagnosis. The continued growth of such cancers usually leads, in the absence of intervention, to invasion, metastasis and death over a relatively short time; for most cancers, these events are not rate limiting. The idea that a small number of events can lead to cancer might be correct, but at death there might be many more, some of which provide a further growth advantage subject to clonal selection. Centrosome abnormalities, the emergence of chromosomal breakages, fusions and bridges, and widespread heterologous translocations characterize this period in the life of most cancers. This state clearly represents a 'mutator phenotype'[53].

A second kind of genomic instability — mutational microsatellite instability (MIN)[52] — is not associated with CIN. Tumours that occur in people with hereditary nonpolyposis colorectal cancer (HNPCC) have greatly elevated (~1,000- fold) rates of specific locus mutations[54]. The inherited mutation occurs in mismatch repair (MMR) genes, most frequently *MSH2* or *MLH1*. A somatic mutation in, or loss of, the remaining normal allele renders the affected cell homozygously defective for MMR. Especially vulnerable is the *TGFBR2* gene, which encodes a receptor in an important signal transduction pathway[55]. Mutations in this receptor are strongly selective for increased growth rate. The number of other events that are necessary for production of a carcinoma cell in HNPCC has not been determined. The cells with homozygous mutations in MMR genes clearly have a 'mutator phenotype', even though they do not show CIN. What CIN and MIN seem to have in common is the ability to increase the rate of transit along the path to clinical cancer.

The view ahead

The genetics of cancer has passed from infancy to maturity in the past century and has brought us to a dazzling, often confusing, view. Cancer cells themselves experience birth, development and death (too often with the patient). In some, karyotypic changes are few, whereas in others there is a bewildering array of abnormalities. Consideration of cancers from many perspectives raises the possibility that the crucial changes on the initiating path to all cancers are few, affect both birth and death processes, and are strongly selected for. In tumours with a single genetic defect, a solitary oncogenic translocation (as seems to be the case in chronic-phase CML), the prospect of developing a successfully targeted therapeutic agent promises to be the greatest. By contrast, developing therapies for the 'multi-hit' tumours will be more challenging, as one agent acting on one target might not be sufficient. On the other hand, the time intervals between multiple hits might be windows of opportunity for preventive agents, in which transition to the next step (such as the second hit in generating the colonic adenomatous polyp) could be delayed or prevented.

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Appendix
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OPINION

Actin' up: RhoB in cancer and apoptosis

George C. Prendergast

RhoB is a small GTPase that regulates actin organization and vesicle transport. It is required for signalling apoptosis in transformed cells that are exposed to farnesyltransferase inhibitors, DNA-damaging agents or taxol. Genetic analysis in mice indicates that RhoB is dispensable for normal cell physiology, but that it has a suppressor or negative modifier function in stress-associated processes, including cancer.

Rho proteins are receiving increasing attention from cancer researchers owing to evidence that they modulate the proliferation, survival, invasion and angiogenic capacity of cancer cells. This family of actin regulatory small GTPases (BOX 1) is not mutated in cancer. However, their altered expression or activity might be crucial to cancer progression and therapeutic responses.

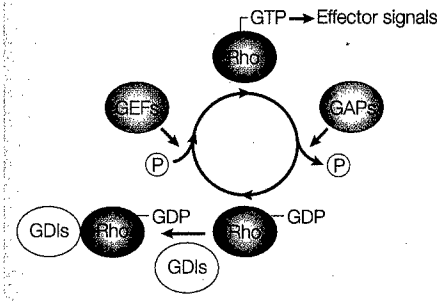
Recent advances indicate that RhoB is a specialized activator of apoptosis in transformed cells. Through a gain-of-function mechanism, RhoB has an important role in mediating the cellular response to farnesyltransferase inhibitors (FTIs). These experimental therapeutics are widely known for their selective effects on neoplastically transformed cells. Although some questions remain about exactly how RhoB alteration fits into the FTI response, many of the biological effects of FTI treatment have been linked to RhoB. Of particular interest, evidence indicates that RhoB is a crucial target for FTI-induced apoptosis. Recently, this role was extended with the finding that RhoB is required for the apoptotic response of transformed cells to DNA damage or taxol. Genetic analysis in mice indicates that RhoB is dispensable for normal cell physiology, but that it limits cancer susceptibility and modifies growth factor and adhesion signalling in transformed cells. What are RhoB's effector mechanisms, and how might they promote apoptosis?

Unique features of RhoB

Rho proteins, which are themselves a subset of the Ras superfamily of isoprenylated small GTPases, can be further divided into subgroups of Rho, Rac and Cdc42 proteins. These regulate a number of cellular processes[1]

Box 1 | Rho cycles: a primer on small GTPase action

Rho proteins are a subfamily of the Ras superfamily of small GTP-binding proteins. Like other members of this superfamily, Rho proteins regulate intracellular signal transduction, acting as switches that cycle between GTP- and GDP-bound states (see figure). In the GTP-bound state, Rho proteins are competent to bind to effector molecules that facilitate downstream signal transmission. This process is shut off by GTP hydrolysis, which is promoted in cells by GTPase-activating proteins (GAPs). In the GDP-bound state, Rho proteins are inactive for signalling, but can be reactivated by GTP exchange. This process is catalysed in cells by guanine nucleotide exchange factors (GEFs), which are themselves activated by upstream signals. Rho GEFs are marked by a common domain known as the DBL domain — named after the *DBL* oncogene, which is the prototype of this class. A third level of regulation is provided by guanine nucleotide dissociation inhibitors (GDIs), which block nucleotide hydrolysis or exchange and which coordinate protein movement between membranes and the cytosol when bound. A large number of Rho GEFs, GAPs and GDIs have been identified in cells, but their physiological roles and specificities are poorly characterized in most cases.



(BOX 2). The Rho subgroup includes the closely related and well-studied RhoA, RhoB and RhoC proteins. These are quite similar to each other in structure (~90% identity) and, in particular, their effector domains are identical. However, as each protein has a different biological role, differences in subcellular localization presumably lead to a partitioning of possible effector interactions and thereby to a differentiation of functions. All Rho proteins regulate actin stress fibres at some level, but how this common property is integrated with their distinct biological functions is not yet understood. One appealing possibility is that Rho proteins regulate various actin-dependent steps in vesicle transport[2]. The possibility that actin and vesicle movement might directly impact cancer biology is no longer as speculative as we might imagine (for example, see REF. 3). As discussed below, RhoB might have a special function in transformed cells, relating to its localization to early endosomes or other intracellular membranes[4–8], and to its role in intracellular transport of cell-surface receptors[9,10].

RhoB has several additional features that are distinct among Rho proteins. First, unlike most small GTPases, which are relatively stable, RhoB is turned over quickly and its synthesis is rapidly upregulated by various growth and stress stimuli[6,11–14]. Second, RhoB is expressed as either geranylgeranylated (RhoB-GG) or farnesylated (RhoB-F) isoforms in cells (BOX 3). Although the significance of this unique feature is uncertain, it is clear that different prenyltransferases are responsible for generating each isoform in cells[15]. Last, although early studies showed that RhoB has a positive role in growth regulation[6,11,15,16], more recent studies indicate that RhoB might also negatively regulate cell proliferation[17–20]. A negative role in growth control and/or TRANSFORMATION would contrast with the positive effects of RhoA and RhoC in these processes[21,22]. However, as RhoB is upregulated by both growth and stress stimuli, it seems more likely to act in a contextual manner. Therefore, we might more accurately describe RhoB as having a modifier function — that is, a function that alters the physiological setting that is required for the operation of certain signalling pathways, as illustrated by the way that integrins dictate the competence of growth factors to stimulate mitogenesis.

RhoB is a crucial FTI target

RhoB was little studied before it became the focus of an intensive investigation into how neoplastic transformation is blocked by FTIs[23,24]. This class of cancer therapeutics was originally developed as a strategy to inhibit Ras function in tumour cells, by blocking the post-translational farnesylation of Ras (which is required for the membrane localization and oncogenic activity of mutant Ras proteins). Early preclinical studies showed that FTIs were exquisitely selective antagonists of Ras-dependent neoplastic transformation, with few, if any, significant effects on untransformed cells (at pharmacologically relevant drug concentrations). However, mechanistic investigations showed that Ras inhibition could not entirely explain their antitransforming properties[23,25]. So, although FTIs inhibit Ras farnesylation, their biological effects can be traced beyond this effect.

The biology, mechanism and applications of FTIs have been considered indepth elsewhere[23,25–30]. Here, I summarize the evidence that prompted a Rho-based model for FTI action[23,24,26]. First, FTIs had little effect on the proliferation of untransformed cells, but they dramatically inhibited the proliferation of cells transformed by oncogenic Ras proteins. These observations were paradoxical, as wild-type Ras proteins require farnesylation, and their function is important for the proliferation of untransformed cells. Second, the kinetics of FTI biology are rapid, outpacing the kinetics of elimination of processed Ras from cells[31]. Last, although FTIs had little effect on the proliferation of untransformed cells, they induced actin stress-fibre formation in those cells[31]. These observations indicated that the FTI response might be mediated by alteration of a short-lived protein that affected actin organization — RhoB fulfilled these criteria[23].

Subsequent investigations led to a genetic proof of the model that RhoB alteration is crucial to the FTI response in Ras-transformed cells[17,18,32]. Unexpectedly, it was found that FTIs caused a gain of function in RhoB, rather than a loss of function, and that this event was sufficient to mediate phenotypic reversion, growth inhibition, cytoskeletal actin reorganization and apoptosis (reviewed in REF. 24). As discussed in more detail below,

Box 2 | Rho proteins have diverse biological roles

Rho proteins regulate a wide spectrum of cellular processes. Members of this family, including the prototypes RhoA, Rac1 and Cdc42, were initially identified as regulators of cytoskeletal actin organization. However, it has become apparent that Rho proteins have diverse cellular functions, which include the regulation of adhesion, motility, vesicle transport, cell-cycle progression, cytokinesis and transcription. This diversity is reflected in the large number of effector signalling pathways that are activated by various Rho proteins, as well as the intimate linkages between them, making functional investigations complex. There is increasing evidence that Rho proteins influence a variety of important processes in cancer, including cell transformation, survival, invasion, metastasis and angiogenesis. Although Rho proteins do not seem to be mutated in cancer cells, their expression is often elevated, indicating that Rho dysregulation promotes malignant phenotypes.

Box 3 | **RhoB-F and RhoB-GG: same or different functions?**

RhoB is post-translationally modified at its carboxyl terminus with either farnesyl (C15) or geranylgeranyl (C20) isoprenyl groups in cells. The ratio of F:GG isoforms can be altered by specific prenyltransferase inhibitors such as farnesyltransferase inhibitors (FTIs) or geranylgeranyltransferase inhibitors (GGTIs). In the presence of a specific FTI, only RhoB-GG can be synthesized; conversely, in the presence of a specific GGTI, only RhoB-F can be synthesized. Do these proteins differ in function? There is some support for this idea[15], but also for overlapping functions[18,19]. A caveat is that the evidence for overlapping functions is based on engineered isoforms that, on overexpression, probably mislocalize. It is not known whether engineered RhoB-F is properly localized[19], as the C-terminal mutation made in this mutant might also affect protein palmitoylation[4]. Engineered RhoB-GG is mislocalized[4], although this mimics the effect of FTI on wild-type RhoB[5]. It will be important to confirm any putative functions of the engineered RhoB-F using *RhoB*$^{-/-}$ cells. Changes in the ratio of F:GG isoforms might alter RhoB localization and therefore function (BOX 5). The role of RhoB as a primary FTI target is not compromised by the possibility of overlapping functions for RhoB: FTIs deplete RhoB-F from cells, so any inhibitory actions that RhoB-F might have are irrelevant in FTI-treated cells (although they might be relevant to GGTI-treated cells). This area deserves further attention, as any differences in function might merely reflects differences in localization.

the gain of function was manifested as an altered pattern of prenylation, elevated expression and subcellular mislocalization. So, FTIs alter RhoB in a radically different manner from Hras (FIG. 1). The relative obscurity of RhoB and the greater complexity of its function in the effects of FTIs continues to prompt investigations into its action. Nevertheless, the necessity of RhoB gain of function in the FTI response has now been established with the recent demonstration of a defective FTI response in mice that are genetically null for the *RhoB* gene[32].

One of the most interesting aspects of RhoB as an FTI target is that it is dispensable for normal physiology in the mouse[20]. Similarly, FTIs have few, if any, discernable effects on mouse physiology (for example, see REF. 33). From these observations, it can be inferred that *any* alteration in RhoB activity induced by FTIs is inconsequential in untransformed cells, as the *RhoB* gene can be entirely deleted without consequence[20]. By contrast, FTI-induced alteration of RhoB has marked effects in Ras-transformed cells. Therefore, it is apparent that, as an FTI target, RhoB has a transformation-selective quality. Of the other FTI targets that might participate in the FTI response, many would seem to be important to normal cell physiology, making it more difficult to invoke their alteration as a way to explain the transformation-selective effects of FTIs (BOX 4).

FTI's ability to accentuate the function of RhoB, rather than to abolish it, relies on the fact that cells express two differently prenylated RhoB isoforms and that 'pure' FTIs do not inhibit geranylgeranyltransferase type I (GGT-I), the enzyme responsible for geranylgeranylating RhoB[15]. Because FTIs do not stop the formation of RhoB-GG, the relative level of RhoB-GG increases as RhoB-F decreases (pre-existing RhoB-F turns over and is not replenished)[15]. Accumulation of RhoB-GG might be promoted further by a forward feedback mechanism that is associated with increased transcription of the *RhoB* gene (S. Sebti, personal communication). This mechanism is uncharacterized, but probably reflects the fact that *RhoB* is autoregulated[34]. Notably, the altered prenylation and expression elicited by FTI are associated with subcellular mislocalization: RhoB-GG becomes separated from the endosomal compartment, where it is normally found[4,5]. Although the exact consequences of mislocalization remain to be established, this event might have important consequences for how FTIs work (BOX 5).

Strikingly, much of the FTI response in cells that are neoplastically transformed by Ras can be mimicked simply by elevating the levels of an engineered RhoB-GG isoform[17]. This isoform was generated by substituting the normal carboxyl terminus of RhoB with that of RhoA, which can be geranylgeranylated but cannot be farnesylated[4]. Expression of this engineered isoform — which mislocalizes, like wild-type RhoB-GG in FTI-treated cells[4] — was sufficient to mediate growth inhibition and apoptosis in human tumour cells as well as in transformed rodent cells[17,18]. By contrast, the RhoB-GG isoform had little effect on untransformed rodent cells, similar to FTI treatment[17].

The conclusion that RhoB-GG elevation is also crucial for the FTI response came from a comparison of the FTI response in genetically distinct populations of MOUSE EMBRYO FIBROBLASTS (MEFs) that were co-transformed by adenovirus E1A and mutant Ras. This combination of oncogenes offers a standard tool for the neoplastic transformation of primary cells. Transformed wild-type MEFs showed a characteristic FTI response in all regards. By contrast, transformed cells that were genetically null for *RhoB* showed a variety of defects in their FTI response. These defects were striking because farnesylation of mutant Ras was inhibited to the same extent in both *Rho*$^{-/-}$ and *Rho*$^{+/-}$ cells. Because FTIs could not elevate

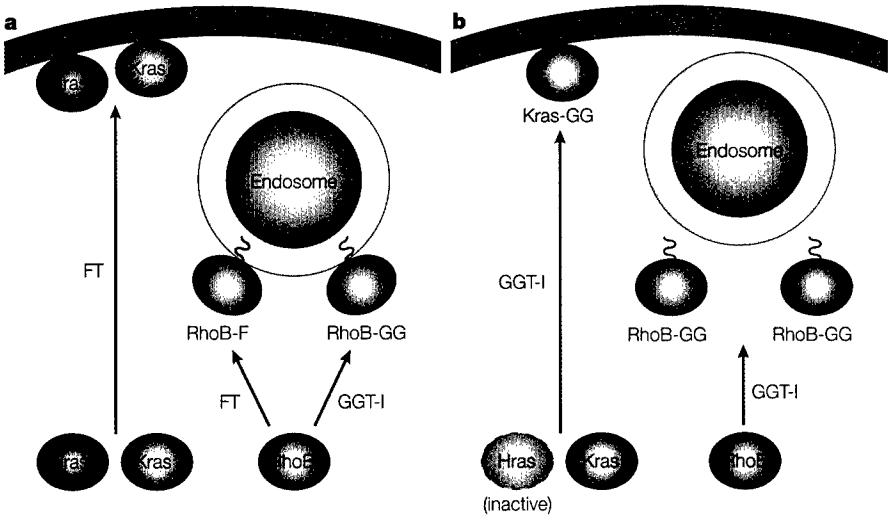


Figure 1 | **How FTIs affect RhoB versus Ras proteins. a** | Newly synthesized precursor proteins are normally modified by farnesyltransferase (FT) or geranylgeranyltransferase type I (GGT-I). **b** | FT inhibitors (FTIs) block the farnesylation of Hras, resulting in a loss of function. By contrast, Kras becomes geranylgeranylated by GGT-I in FTI-treated cells, remaining membrane-associated and active. FTIs also block formation of RhoB-F, but RhoB-GG accumulates owing to geranylgeranylation of all newly synthesized protein by GGT-I. The shift in prenylation pattern of RhoB is associated with elevated expression and mislocalization of RhoB-GG away from its normal endosomal location in cells.

Box 4 | **Proposed FTI targets**

The best-studied farnesyltransferase inhibitor (FTI) targets so far are the Ras proteins (Hras, Kras and Nras) and RhoB. So far, RhoB is the only FTI target that has been critically assessed for its involvement in the FTI response using a mouse gene-knockout system. Kras and Nras become geranylgeranylated by geranylgeranyltransferase type I (GGT-I) in FTI-treated cells, where they can continue to function in signal transduction and neoplastic growth. By contrast, Hras cannot be prenylated in FTI-treated cells, so it is inactivated (FIG. 1). Some investigators have proposed that a loss of Hras function in tumour cells driven by mutant Kras, Nras or other oncogenes might underlie the antiproliferative or proapoptotic effects of FTIs in such settings. *Hras* knockout mice, which are viable[60], offer an excellent model to critically assess this hypothesis. Many other farnesylated proteins have been proposed as candidate FTI targets, but whether inhibiting the farnesylation of these proteins is involved in mediating the transformation-selective properties of FTIs has yet to be established. A partial list of these candidate targets includes the lamin A and B proteins; the protein phosphatases PTP1 and PTP2; the peroxisomal protein PXF; the molecular chaperone HDJ-2; inositol trisphosphate 5′-phosphatase; the small GTPases RhoD, RhoE/Rnd and Rheb; the skeletal muscle α- and β-phosphorylase kinases; and the centromere-binding proteins CENPE and CENPF.

RhoB-GG in the absence of the *RhoB* gene, the different response of $RhoB^{-/-}$ cells to FTI treatment was attributed to the absence of RhoB-GG[32]. This study also addressed the caveats of previous studies that used an engineered RhoB-GG isoform to establish sufficiency[17,18], by confirming that the endogenous wild-type RhoB-GG isoform was crucial to the FTI response. Of particular note, this study identified a necessary role for RhoB-GG in cytoskeletal actin reorganization and apoptosis induced by FTI. The apoptotic defect was vital, because it compromised the drug's antitumour activity *in vivo*[32]. So, the most interesting feature of FTIs — their ability to selectively activate apoptosis in transformed cells — involves a gain of RhoB function that is based on an alteration or accentuation of its activity.

RhoB in apoptosis by DNA damage

Recent evidence indicates that RhoB is required for the apoptotic response of transformed cells to DNA-damaging agents or to taxol[35]. This conclusion was derived from a study that used the same genetically defined MEFs as the FTI study. Untransformed primary MEFs respond to DNA damage by undergoing cell-cycle arrest and chromosomal repair. These cells are highly resistant to apoptosis, presumably because they can appropriately arrest and repair any damage. In contrast, MEFs that are transformed by E1A and mutant Ras are highly susceptible to DNA-damage-induced apoptosis, following a transient cell-cycle arrest, presumably because they cannot engage an appropriate arrest-and-repair response[36]. *RhoB* was dispensable for the cell-cycle arrest response of MEFs that were exposed to γ-irradiation, which causes DNA strand breaks, or to doxorubicin, which indirectly causes strand breakage by inhibiting DNA topoisomerase. By contrast, transformed cells lacking *RhoB* did not undergo apoptosis after DNA damage; this defect was complemented by ectopic expression of *RhoB*[35]. Consistent with previous work, RhoB was also found to be required for FTIs to sensitize cells to DNA-damage-induced cell death[37,38]. In support of these findings are reports that genotoxic stress can stimulate transcription of *RhoB*[12], and that *RhoB* overexpression can sensitize certain cells to apoptosis by DNA-alkylating agents[39]. Notably, *RhoB* deletion did not affect the susceptibility of transformed MEFs to apoptosis by the broad-spectrum kinase inhibitor staurosporine[35]. So, RhoB was not generally required for apoptosis. However, loss of RhoB also diminished the apoptotic susceptibility to taxol[35]. Together, these findings revealed a significant role for RhoB in apoptosis that is induced by agents that damage DNA in transformed cells or that interfere with microtubules — actions that encompass two classes of cancer chemotherapeutic drugs.

Significantly, the defective apoptotic response of transformed $RhoB^{-/-}$ cells improved their long-term survival after DNA damage *in vitro*. Moreover, this defect promoted cell survival in tumours that were challenged by γ-irradiation in a tumour XENOGRAFT ASSAY[35]. As DNA-damaging agents and taxanes, such as taxol, represent an important part of the arsenal of the clinical oncologist, these observations indicated that RhoB might be involved in many therapeutic responses in cancer. Furthermore, these results indicated that loss or inactivation of RhoB, or its effector signalling pathways, might contribute to the development of radioresistance or chemotherapeutic resistance in cancer.

Dose-response studies showed that DNA-damage-induced apoptosis was initiated in $RhoB^{+/-}$-transformed cells after a transient arrest in the G2/M phase of the cell cycle, consistent with previous observations[36]. In $RhoB^{-/-}$ cell populations, however, there was a progressive increase in the number of cells arrested in the G2/M phase, without any subsequent apoptosis. As noted above, RhoB loss also compromised cell death by taxol, which is believed to induce death by arresting the microtubule-based spindle apparatus at mitosis[35]. Together, these observations indicate that RhoB might participate in an apoptotic pathway that is engaged following a terminal block in mitosis. This faulty apoptotic response following G2/M arrest was not related to p53-mediated cell-cycle arrest[36]. Therefore, as summarized in FIG. 2, it can be concluded that RhoB does not participate in activation of the DNA-damage-response pathway, but instead acts downstream or in parallel to this pathway as a death effector or modifier, respectively.

RhoB as a suppressor or modifier

$RhoB^{-/-}$ mice do not have any apparent defects in development, fertility or wound healing[20]. However, there is evidence that RhoB might be necessary for appropriate cell adhesion and growth control during cell

Box 5 | **Does RhoB interfere with Rho-transforming signals?**

RhoB can mediate growth inhibition and apoptosis in neoplastic cells. These functions contrast with other Rho proteins, such as RhoC, which is tumorigenic[22,59,61,62]. The effector domains of RhoA, RhoB and RhoC are virtually identical, and in several cases 'RhoA effectors' have been confirmed to interact with RhoB. Moving RhoB between different cellular compartments, as FTIs seem to do, might allow RhoB to compete for effector functions that are normally unavailable. Competition for Rho guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) or guanine nucleotide dissociation inhibitors (GDIs) is also conceivable, widening the scope of RhoB action to include many Rho, Rac or Cdc42 proteins, which share many of these factors. In this way, RhoB could act as a dominant inhibitor that disrupts or counters Rho signals involved in proliferation, survival or transformation (RhoB-GG effects elicited by FTIs might represent one mechanism for how this can be done). Two predictions of such a dominant inhibitory model are that FTIs should inhibit transformation by geranylgeranylated Rho proteins, such as Rac1 or RhoC, and that stresses that elicit RhoB-dependent apoptosis, such as genotoxic stress, should lead to elevation and altered localization of one or both RhoB isoforms.

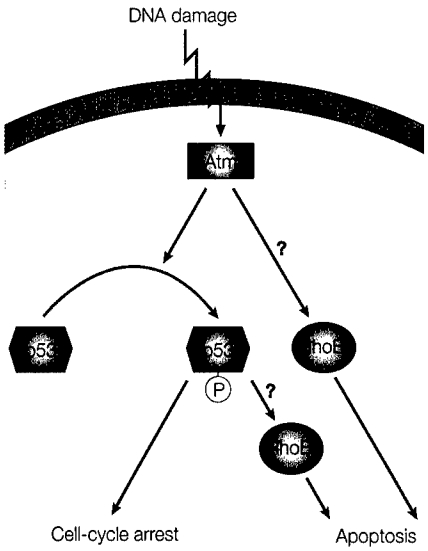


Figure 2 | **RhoB is needed for apoptosis of transformed cells after DNA damage.** RhoB is dispensable for detecting DNA damage and for upstream signals that elicit cell-cycle arrest, such as activation of Atm or p53. RhoB is required in an apoptotic effector or modifier pathway, which might become activated in the G2/M phase of transformed wild-type cells, presumably downstream of or in parallel to p53.

stress. For example, primary *RhoB*$^{-/-}$ MEFs showed a defect in motility on fibronectin. As no defects in wound healing were observed *in vivo*, the defect observed in MEFs was interpreted to be conditional on stresses that are caused by *in vitro* culture (for example, see REF. 40). A role in motility has also been indicated by RhoB upregulation in motile neural crest cells during avian development[41]. No defects in MEF attachment or spreading were observed, but neoplastic transformation of *RhoB*$^{-/-}$ MEFs by adenovirus E1A and mutant Ras led to a marked reduction in the rate of attachment and spreading relative to *RhoB*$^{+/-}$ MEFS.

RhoB loss also accentuated proliferative responses in transformed cells. Consistent with *in vitro* observations, *RhoB*$^{-/-}$ cells formed tumours more efficienctly than *RhoB*$^{+/-}$ cells after injection into the intraperitoneal cavity of mice. The stromal environment was important to this effect, because no differences in tumour formation efficiency were observed if cells were injected subcutaneously[32,35]. More significant support for a suppressor or negative modifier role was evidenced by the elevated rate of papilloma formation in *RhoB*$^{-/-}$ mice subjected to a classical skin carcinogenesis assay, which involves initiation with the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) and promotion with phorbol ester[20]. The findings that RhoB suppresses tumorigenesis and restrains the transformed characteristics of neoplastic cells were consistent with the evidence that the antitransforming effects of FTIs are mediated by a gain of function in RhoB. In summary, a self-consistent picture emerges from the FTI investigations and the mouse genetic analysis, indicating that RhoB is a suppressor or negative modifier that operates under cell stress conditions, including stresses caused by neoplastic transformation.

How does RhoB act?

RhoB influences adhesion and proliferation[24] in transformed cells, but its most interesting feature might be its ability to cause transformed cells to undergo apoptosis. Because of the identity between the effector domains of RhoA and RhoB, many putative RhoA effectors that have been identified might actually be relevant to RhoB action (BOX 5). Two such effector molecules are the protein kinase C-related kinase (Prk) and the actin–microtubule regulatory protein mouse Diaphanous (mDia)[1]. Prk kinases, including Prk1 and Prk2, have been implicated in Rho-dependent processes, such as cell motility and cell polarity. mDia proteins, including mDia1 and mDia2, are Rho-binding adaptor proteins that coordinate the organization of actin filaments and microtubules. The cell-survival kinase AKT and the transcription factor NF-κB might also be effectors of RhoB, as each is influenced by FTIs and/or RhoB.

The interest in the Rho effector kinases Prk1 or Prk2 is based on the possibility that they might link the activities of RhoB and Akt. RhoB can recruit Prk1 and Prk2 to early endosomes for vesicle transport[9,10]. Prk kinases further recruit the Akt regulatory kinase Pdk1 to generate a ternary complex that depends on RhoB for its formation[42]. This complex might be relevant to Akt regulation by RhoB, based on the suggestion that Prk–Pdk interactions are required for phosphorylation of the Akt regulatory site Ser473 (REF. 43). Although this biochemical linkage is speculative, it offers a possible explanation for the finding that FTIs and RhoB-GG can regulate Akt activity[45]. In this model, mislocalization of the RhoB-GG–Prk–Pdk1 complex in certain FTI-treated cells might lead to inhibition of Akt and subsequent cell death (FIG. 3).

FTIs suppress Akt activity in ovarian and pancreatic carcinoma cells[44]. Similarly, FTIs or RhoB-GG suppress Akt activity in COS cells or MCF-7 breast carcinoma cells[45]. Lastly, FTIs suppress Akt activity in a RhoB-dependent manner in retinal endothelial cells (L. Benjamin, personal communication). These effects are tissue specific, as neither FTIs nor RhoB-GG suppress Akt activity in transformed fibroblasts[45]. However, activated (*N*-myristoylated) Akt can suppress apoptosis that would normally be induced by FTIs in transformed fibroblasts or ovarian carcinoma cells[44,46], in support of the notion that RhoB-GG might elicit apoptosis, at least in part, by causing mislocalization of Akt and suppressing its activity at appropriate sites in the cell. Further investigations are clearly required to determine how relevant the RhoB–Prk–Pdk1 linkage might be in these settings, and the extent to which RhoB-dependent apoptosis is founded on Akt suppression. The availability of *Akt*$^{-/-}$ cells and mice would help to resolve this issue.

Another cell-survival regulator that might mediate downstream signalling from RhoB is NF-κB (FIG. 3), based on the recent finding that RhoB can suppress NF-κB by interfering with IκB turnover[47]. The biochemical linkage between RhoB and IκB regulation has not been defined; moreover, effects of RhoB on NF-κB might be tissue specific, because FTI-induced apoptosis was not found to be causally linked with NF-κB modulation in one study[46]. Nevertheless, given the likelihood that Rho proteins regulate NF-κB[48,49], including in response to genotoxic stress[50], further investigations in this area are warranted.

Glossary

AKT
Also known as protein kinase B. This is a serine/threonine protein kinase activated by the phosphatidylinositol-3-OH kinase pathway that activates survival responses.

ANAPHASE-PROMOTING COMPLEX
(APC). This complex triggers chromosome separation at the end of metaphase in mitosis. It is inactivated by checkpoint pathways that are triggered by damage to the mitotic spindle or by kinetochore problems that cause chromosomes to be released from the spindle.

MOUSE EMBRYO FIBROBLASTS
(MEFs). These cells are widely used to characterize the gross effects of gene deletion because they can be readily cultured from mice, including knockout mice that do not survive to term.

NF-κB
A heterodimeric transcription factor that is regulated by a variety of extracellular stimuli, including many that regulate cell survival.

TAXOL
A chemotherapeutic agent that kills cancer cells in mitosis by stabilizing microtubules.

TRANSFORMATION
The processes through which normal cells acquire malignant character.

XENOGRAFT ASSAY
A tumour-formation assay in which heterologous tumour cells are grown in an immunologically compromised mouse, or other animal, often under the skin.

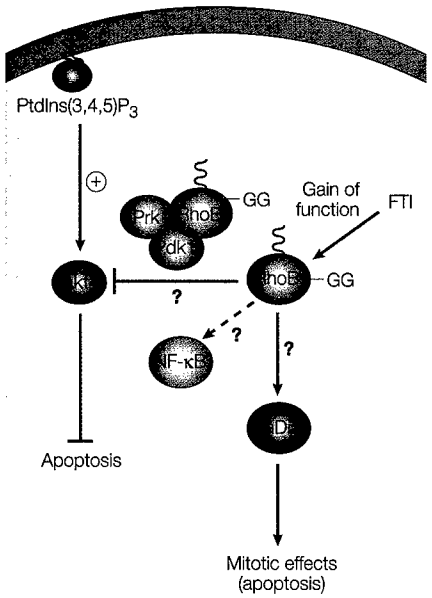


Figure 3 | **Potential RhoB death effector pathways.** RhoB-GG levels are elevated by FTIs and this suppresses Akt (which is activated by phosphatidylinositol(3,4,5) trisphosphate ($PtdIns(3,4,5)P_3$) in epithelial and endothelial cells. Akt activity might be modulated by a ternary complex that contains RhoB, a Prk effector kinase, and the Akt regulatory kinase Pdk1 (REFS 42,43). The Rho effector mDia can interact with RhoB and might also contribute to apoptotic induction by DNA-damaging agents and taxol (not shown), as well as FTIs; all of these stimuli might activate apoptosis from the G2/M phases of the cell cycle. Although it has not been linked to RhoB-dependent apoptosis, NF-κB should be investigated further in this regard as it might be regulated by RhoB (see text).

Another effector molecule that might be relevant to apoptotic signalling by RhoB is mDia (FIG. 3). This adaptor protein coordinates the orientation of actin fibres and microtubules in a Rho-dependent fashion, and might be involved in mediating communication between the mitotic spindle and the cleavage furrow during mitosis[51]. Although this is speculative, mDia has appeal as a potential RhoB death effector based on the unexpected findings that apoptosis induced by taxol involves RhoB, and that apoptosis induced by DNA damage might be initiated during the M phase of the cell cycle[35]. Potential interaction between RhoB-GG and mDia might also address the marked effects of FTIs within the G2/M phases of the cell cycle in cancer cells[52–54], including interesting effects on spindle-formation patterns[55]. A tumour-suppressor or negative-modifier function for a RhoB–mDia pathway is intriguing in this context, especially in light of the recent description of a new actin-dependent and ANAPHASE-PROMOTING COMPLEX (APC)-independent checkpoint for spindle alignment in fission yeast[56]. This checkpoint apparently monitors actin–microtubule interactions. Interestingly, its engagement involves activation of the *ATF1* gene, which is homologous to the mammalian transcription factor ATF2 that regulates *RhoB* transcription[57]. This speculative checkpoint mechanism might be relevant to RhoB-induced apoptosis, based on the role of mDia in coordinating actin–microtubule organization, the ability of DNA-damaging agents, taxol and FTIs to disrupt M phase events, and the ability of the new checkpoint to elicit cell death.

Implications

RhoB is dispensable for physiological cell death, but important for apoptosis that is induced in transformed cells by several types of cancer therapeutics. This specialized role differs from that of most 'classical' apoptosis regulators, which act in both untransformed and malignantly transformed cells. One implication of Rho's involvement in cancer-cell-specific apoptosis concerns the clinical application of FTIs. As I have argued elsewhere[58], FTIs might be useful to treat Rho-driven cancers, such as inflammatory breast cancer or melanoma[22,59], if RhoB-GG counters or disrupts Rho-transforming signals, as hypothesized[58] (BOX 5). As RhoB-GG can suppress Akt activity in some settings, FTIs might also be useful against cancers in which Akt activation is implicated, such as prostate cancer. A second implication of recent work is a possible role for RhoB in a DNA-damage checkpoint or death effector pathway, perhaps linked to the actin–microtubule organization and/or vesicle transport. This area opens new vistas in the areas of apoptotic signalling, cancer pathophysiology, tumour progression and therapeutic responses. Given its role in stress responses, it is conceivable that RhoB might be relevant to other pathological states involving cell stress, such as inflammatory responses or reperfusion injury. Last, recent work implies that inactivation of RhoB or its effectors could generate drug resistance or radioresistance in cancer cells. Further investigations into how RhoB suppresses transformation and activates cancer-cell suicide might produce new insights into the roots of neoplasia, cancer-cell apoptosis, and improved methods to treat cancer patients in a more specific manner.

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Appendix
Prendergast, G.
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Reversion of RhoC GTPase-induced Inflammatory Breast Cancer Phenotype by Treatment with a Farnesyl Transferase Inhibitor[1]

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Abstract

Inflammatory breast carcinoma (IBC) is a highly aggressive form of locally advanced breast cancer that has the ability to invade and block the dermal lymphatics of the skin overlying the breast. In a previous series of studies, our laboratory identified overexpression of RhoC GTPase in >90% of IBCs (K. L. van Golen *et al.*, Clin. Cancer Res., *5:* 2511–2519, 1999) and defined *RhoC* as a mammary oncogene involved in conferring the metastatic phenotype (K. L. van Golen *et al.*, Cancer Res., *60:* 5832–5838, 2000). RhoC GTPase is involved in cytoskeletal reorganization during cellular motility. Farnesyl transferase inhibitors (FTIs) were previously shown to be effective in modulating tumor growth in Ras-transformed tumor cells. Recently, studies have focused on RhoB as a putative non-Ras target of FTI action. In the present study, we assessed the effect of the FTI L-744,832 on RhoC-overexpressing IBC and RhoC-transfected human mammary epithelial (HME-RhoC) cells. Treatment of the SUM149 IBC cell line and HME-RhoC transfectants with the FTI L-744,832 led to reversion of the RhoC-induced phenotype, manifested by a significant decrease in anchorage-independent growth, motility, and invasion. Although RhoC expression and activation were not affected, RhoB levels were increased by FTI treatment. Transient transfection of geranylgeranylated RhoB (RhoB-GG) into the same cells reproduced the effects of the FTI, thus suggesting that FTI-induced reversion of the RhoC phenotype may be mediated by an increase in RhoB-GG levels. These data provide direct evidence that FTIs may find use in the clinic when directed against RhoC-overexpressing tumors and suggest appropriate biological markers to evaluate during FTI treatment.

Introduction

The term IBC[3] was first coined in 1924 by Drs. Lee and Tannenbaum to describe a phenotypically distinct form of locally advanced breast cancer (LABC) (1, 2). IBC is a fast-growing, highly invasive, and metastatic form of LABC, which is clinically characterized by primary skin changes (1–4). These primary skin changes are the result of blockage of the dermal lymphatics of the skin overlying the breast resulting in edema, *peau d' aurange,* and nipple retraction (1–4). At the time of diagnosis, nearly all tumors have spread to the regional lymph nodes and on close inspection, more than one-third of patients have gross distant metastases (1–4). Despite aggressive multimodality treatments, the 5-year disease-free survival rate for women with IBC is <45%, making IBC the deadliest form of breast cancer (1–4).

During investigation of the genetic mechanisms responsible for the unique IBC phenotype, our laboratory identified overexpression of RhoC GTPase in >90% of IBCs (5). RhoC GTPase is a member of the Ras-homology family of small GTP-binding proteins and is responsible for cytoskeletal reorganization during cellular motility (6–10). RhoC belongs to a highly homologous subfamily comprised of RhoA, RhoB, and RhoC (1,1). Although these family members have >90% sequence homology to one another, their roles in the cell are distinct (11). To determine the contribution of RhoC GTPase overexpression to the IBC phenotype, we generated stable RhoC-overexpressing HME cell lines (HME-RhoC) (12). The HME-RhoC clones nearly recapitulated the invasive features of the IBC phenotype. Specifically, the cells grew under anchorage-independent conditions and produced tumors when orthotopically injected into athymic nude mice (12–14). The cells were highly motile and invasive and produced conditioned medium rich in pro-angiogenic cytokines *in vitro* (12–14). Taken together, these data demonstrate that overexpressed, active *RhoC GTPase* is a mammary oncogene leading to advanced disease.

Regulation of the GTPase activity of both the Ras and the Rho proteins is achieved through interactions of GAPs, GDIs, GDF, and GEFs (15, 16). RhoA, RhoC, and a fraction of RhoB

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[3] The abbreviations used are: IBC, inflammatory breast cancer; FTI, farnesyl transferase inhibitor; HME, human mammary epithelial (cells); FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR; PI, propidium iodide; RhoB-GG, geranylgeranylated RhoB; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione *S*-transferase; PI3K, phosphatidylinositol 3′-kinase; β-gal, β-galactosidase; GAP, GTPase-activating proteins; GDI, GDP-dissociation inhibitor; GDF, GDI dissociation factor; GEF, guanine nucleotide exchange factor; SEM, scanning electron microscopy.

are geranylgeranylated, and the remaining portion of RhoB is farnesylated (17–19). For the Rho proteins to enter the GDP/GTP cycle they must be transported and localized to the membrane (19, 20). GTP binding produces a conformational change in the GTPase, thereby allowing interaction with downstream effector proteins (15, 21). Hydrolysis of GTP to GDP by the intrinsic Rho GTPase activity modulates the interaction with the effector protein (22, 23). The GTPase activity is greatly increased by activated GAPs, thus leading to increased hydrolysis of GTP (24). The entire process is balanced by the GDIs, which prevent GDP dissociation by binding to the prenylation group of the GTPase and sequestering the complex in the cytoplasm (25). The GTPase is subsequently liberated from the GDI by GDFs, closing the cycle (25).

In light of evidence demonstrating that FTIs can target non-Ras molecules, such as the RhoB protein, and recent work suggesting that RhoB alterations, specifically, the accumulation of RhoB-GG, may interfere with transforming Rho signals, we sought to test the effect of FTIs on RhoC-transformed breast cells.

Materials and Methods

Cell Culture. Cell lines were maintained under defined culture conditions for optimal growth in each case as described previously (26–28). E6/E7 immortalized HME cells (29) were grown in 5% FBS (Sigma Chemical Co., St. Louis, MO)-supplemented Ham's F-12 medium (JRH BioSciences, Lenexa, KS) containing insulin, hydrocortisone, epidermal growth factor, and cholera toxin (Sigma Chemical Co.). Stable HME transfectants containing either the human *RhoC GTPase* or control *β-gal* genes were maintained in the described medium supplemented with 100 μg/ml hygromycin (LifeScience Technologies; Gaithersburg, MD) as described previously (12, 13). The SUM149 IBC cell line was grown in 5% FBS-supplemented Ham's F-12 medium containing insulin and hydrocortisone. The HME cells were characterized as being keratin 19 positive, thus ensuring that they are from the same differentiation lineage as the SUM149 IBC tumor cell line. For FTI treatment, actively growing cells were treated with 25 μM FTI L-744,832 and harvested 48 h later. Cell viability was assessed prior to assays using a trypan blue exclusion assay. Harvested cells were washed in 10 ml of HBSS (LifeScience Technologies). A 100-μl aliquot was taken, diluted 1:1 with prediluted trypan blue (Sigma Chemical Co.), and counted on a hemacytometer.

Transient transfections were performed by growing cells in 100-mm plates until reaching 50% confluence. Expression constructs for wild-type RhoB, RhoB-GG, and a geranylgeranyl-deficient RhoB mutant were generated as described previously (30–32). The RhoB containing vectors or a vector control were introduced into the cells using FuGene6 transfection reagent (Roche, Indianapolis, IN) as described previously (12). Transient transfectants were used in biological assays 24 h after transfection.

Anchorage-independent Growth and Focus Formation. For anchorage-independent growth assays, a 2% stock of sterile low-melt agarose was diluted 1:1 with 2× MEM. Further dilution to 0.6% agarose was made using 10% FBS-supplemented Ham's F-12 medium complete with growth factors, and 1 ml was added to each well of a six-well plate as a base-layer. The cell layer was then prepared by diluting agarose to 0.3% and 0.6% with 10^3 cells (either untreated or 25 μM FTI L-744,832 for 24 h) in 2.5% FBS-supplemented Ham's F-12/1.5 ml/well. A 1-ml layer of medium was maintained on top of the agar to provide nutrients and, in the case of the treated cells, additional inhibitor. Colonies ≥100 μm in diameter were counted after a 2-week incubation at 37°C in a 10% CO_2 incubator.

A modified focus formation assay was performed by harvesting treated and untreated cells and plating at dilutions of 1000, 500, and 100 cells/35-mm dish. The cells were then cultured for 2 weeks at 37°C in a 10% CO_2 incubator. The plates were washed with 10 ml of PBS, fixed for 10 min with ice-cold methanol, and stained for 10 min with 2% methylene blue in 50% ethanol, and visible foci were counted.

Western Blot and RhoC Activation Analysis. Proteins were harvested from cell cultures using radioimmunoprecipitation assay buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 0.3 mg/ml aprotinin; Sigma Chemical Co.) Ten-μg aliquots were mixed with Laemmli buffer, heat-denatured for 3 min, separated by SDS-PAGE, and transferred to nitrocellulose. Nonspecific binding was blocked by overnight incubation with 2% powdered milk in Tris-buffered saline with 0.05% Tween 20 (Sigma Chemical Co.). Immobilized proteins were probed using antibodies specific for RhoC GTPase (33), or RhoB GTPase (Cytoskeleton Inc. Denver, CO). Protein bands were visualized by ECL (Amersham-Pharmacia Biotech, Piscataway, NJ).

A RhoC activation assay was performed as described previously (34, 35). Cells grown to 40% confluence were incubated in the presence or absence of 25 μM FTI L-744,832 for 24 h. Proteins were harvested using GST-FISH buffer (34) and were centrifuged. The supernatant was mixed with a slurry of GST-rhotekin fusion protein bound to glutathione-sepharose beads. Only GTP-bound Rho binds to the GST-rhotekin fusion protein. The mixture was centrifuged, separated by SDS-PAGE, transferred to nitrocellulose and probed using a RhoC-specific antibody (33). Protein bands were visualized by ECL and exposed to Hyperfilm (Amersham).

Semiquantitative RT-PCR. Total RNA was harvested from actively growing cells at 50% confluence using Trizol Reagent (Life Technologies), and cDNA was made using the AMV-reverse transcriptase kit (Promega, Madison, WI). RhoC and RhoB transcripts were PCR amplified from aliquots of cDNA using a 1:100 dilution of Rho-specific primers mixed with GAPDH primers. PCR products were then separated on a 1.2% TAE-agarose gel and were visualized by ethidium bromide. The relative intensity of the Rho and GAPDH bands was measured using an Alpha Imager 2200 (Alpha Innotech Co., San Leandro, CA).

Motility and Invasion Assays. Random motility was determined using a gold-colloid assay (36). Gold colloid was layered onto glass coverslips and placed into 6-well plates. Cells were seeded onto the coverslips and allowed to adhere for 1 h at 37°C in a CO_2 incubator (12,500 cells/3 ml in serum-free medium). To stimulate the cells, the serum-free

medium was replaced with 5% FBS containing Ham's F-12 supplemented with growth factors and allowed to incubate for 3 h at 37°C. The medium was aspirated and the cells fixed using 2% gluteraldehyde (Sigma Chemical Co.). The coverslips were then mounted onto glass microscope slides and areas of clearing in the gold colloid corresponding to phagokinetic cell tracks were counted.

The invasion assay was performed as described previously with minor modification (37). A 10-μl aliquot of 10 mg/ml Matrigel (BD Biosciences, Bedford, MA) was spread onto a 6.5-mm Transwell filter with 8 μm pores (Costar, Corning, NY) air-dried in a laminar flow hood, and reconstituted with a few drops of serum-free medium. The lower chamber of the Transwell was filled with either serum-free or serum-containing media. Cells were harvested and resuspended in serum-free medium with 0.1% BSA at a concentration of 3.75×10^5 cells/ml, and 0.5 ml was added to the top chamber. The chambers were incubated for 24 h at 37°C in a 10% CO_2 incubator. The cell suspension was aspirated, and excess Matrigel was removed from the filter using a cotton swab. The filters were then cut away from the Transwell assembly and fixed, gel side down, with methanol to a glass microscope slide, stained with H&E, and 20 random ×40 magnification fields were counted. The number of cells that had invaded into the serum-free medium-containing lower chambers were considered background and were subtracted from the number of invaded cells in the serum-containing samples.

Statistical analysis was performed using a two-tailed Student *t* test.

Apoptosis Assay. Cytofluorometric analysis of cell cycle distribution and apoptosis was performed by PI staining of nuclei as reported previously (38, 39). Briefly, cells were treated with 25 μM FTI L-744,832 (Merck) alone, 10 μM LY294002 (Calbiochem, San Diego, CA) alone, or a combination of both FTI L-744,832 and LY294002. Untreated and treated cells, 1×10^6, were harvested from 35-mm wells, washed once with ice-cold PBS (Fisher Scientific, Pittsburgh, PA) and pelleted; supernatants were removed and 500 μl of PI-hypotonic lysis buffer [0.1% sodium citrate, 0.1% Triton X, 100 μg/ml RNAse type I-A, 50 μg/ml PI (SIGMA)] were added. Samples were analyzed by flow cytometry after a 20-min incubation at 25°C.

Rhodamine-Phallodin Staining of Actin Filaments. Visualization of actin filaments was accomplished by staining the cells with a rhodamine-conjugated phallotoxin. Briefly, cells were grown on glass coverslips for 48 h and washed with PBS followed by fixation with 1:1 ice-cold acetone and methanol. After a 30-min incubation in PBS containing 1% BSA, 5 μl of methanolic rhodamine-phalloidin stock (Molecular Probes, Eugene, OR) were added to each coverslip and allowed to stain for 20 min at room temperature. After repeated washing with PBS, the coverslips were mounted onto glass microscope slides using Gel/Mount (Biomedia Co., Foster City, CA). Cells were visualized under a Zeiss scanning laser confocal microscope equipped with a 573-nm fluorescence filter.

SEM. Cells (12,000) were fixed with buffered 2.5% glutaraldehyde for 1 h, rinsed, and post-fixed for an additional hour with buffered osmium tetroxide. After dehydration in ascending strengths of ethanol, the cells were critical-point dried, mounted onto standard SEM stubs, and gold-sputter coated. They were viewed using an AMRAY 1000-B Scanning Electron Microscope.

Results

Effect of FTI Treatment on RhoC-overexpressing Breast Cells. The ability of cells to grow in soft agar is a hallmark of malignant transformation (40). Previously, we found that RhoC overexpression led to the growth of mammary epithelial cells under anchorage-independent conditions (12, 14). As demonstrated in Fig. 1*A*, treatment of RhoC-overexpressing HME cells and the SUM149 IBC cell line with 25 μM FTI L-744,832 resulted in a significant decrease in anchorage-independent growth. Although the HME-β-gal control-transfected cells did not readily grow under anchorage-independent conditions, they were slightly affected by FTI treatment. The 80% decrease in anchorage-independent growth of the Rho-expressing cells did not correlate with a decrease in monolayer growth rate as determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (data not shown). It has been suggested that FTI treatment sensitizes cells to apoptotic death on treatment with the PI3K inhibitor LY294002 (31). However, in our system, we did not observe an increase in apoptosis in cells treated with FTI L-744,832 alone, LY294002 alone, or a combination of the two, as has been observed for Ras-transformed cells (data not shown).

To evaluate the effect of FTI treatment on RhoC-mediated cellular motility, we assessed the same treated cell lines in colloidal-gold random motility assay. Cells were seeded onto glass coverslips overlayed with a gold colloid and stimulated with serum to induce motility. Discernable and quantifiable tracks were left as the cells moved and phagocytized the gold colloid. At 24 h after stimulation, the SUM149 and HME-RhoC cells treated with FTI L-744,832 were 1.8- to 2-fold less motile than their untreated counterparts (Fig. 1*B*). Both the HME-β-gal control and the MCF10AT c1 (an MCF10A clone transfected with a constituitively active Ras; Ref. 41) were unaffected by FTI treatment. As determined by a trypan blue dye exclusion assay, the reduction in cell motility was not caused by a decrease in the number of viable cells (data not shown).

As shown in Fig. 1*C*, when the FTI-treated cells were tested for their ability to invade through a Matrigel-coated filter in response to a chemoattractant, it was found that the SUM149 and HME-RhoC cells were 2-fold less invasive than the untreated cells. Again, the control cell lines, HME-β-gal and MCF10AT c1, were unaffected by FTI treatment.

Taken together, these data suggest that treatment of RhoC-overexpressing cells with a FTI leads to the inhibition of RhoC-mediated anchorage-independent growth, motility, and invasion without significantly affecting cell growth or viability.

As demonstrated in Fig. 2, *C* and *E*, rhodamine-phallodin staining for actin filaments shows a highly organized and polarized cytoskeleton in the RhoC-overexpressing cells. These actin bundles are lost or diminished on treatment with

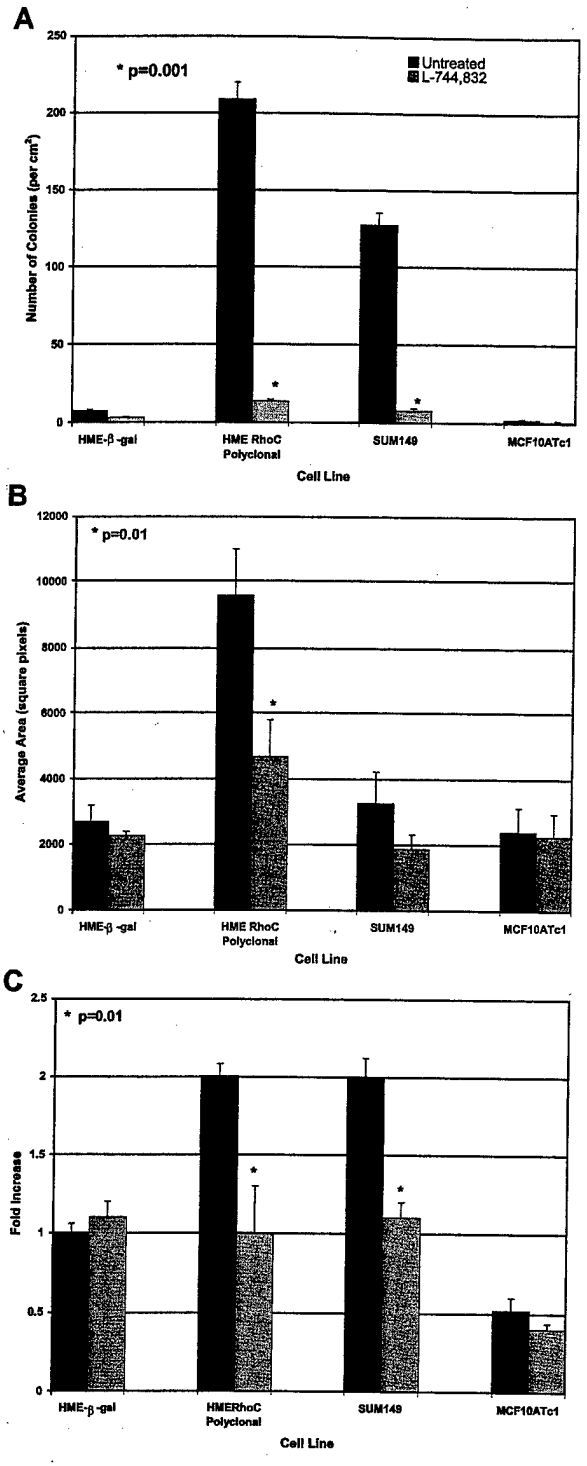

Fig. 1. *A*, comparison of anchorage-independent growth of untreated and FTI L-744,832 treated cell lines in 0.6% soft agar. The growth of RhoC overexpressing cell lines, HME-RhoC and SUM149, were significantly inhibited ($P = 0.001$) by FTI treatment, which suggests a reversion of malignant transformation. *B* demonstrates a significant reduction in the motility of FTI-treated HME-RhoC cells. A reduction in motility, although not significant, was also observed for the SUM149 cells. A significant reduction ($P = 0.01$) in the ability to invade through a Matrigel-coated filter was also observed for the HME-RhoC and SUM149 cells (*C*). None of the effects that resulted from treatment with FTI could be attributed to a decrease in viable cells, as determined by trypan blue dye-exclusion assays or by apoptosis assays.

FTI L-744,832 (Fig. 2, *D* and *F*). Numerous focal adhesions were visible on the periphery of the treated cells. However, loss of cytoskeletal polarity led to morphological changes towards a rounded shape, as demonstrated by laser scanning confocal microscopy and scanning electron microscopy (Fig. 2, *G–J*). The morphology of the control HME-β-gal cells was also similarly affected, albeit to a lesser degree, by FTI treatment, as these cells became dissociated and flattened (Fig. 2, *A* and *B*).

Rho Protein Levels Increase as a Result of FTI Treatment. To determine the effect of FTI treatment on RhoC expression, we performed semiquantitative RT-PCR and Western blot analysis. As shown in Fig. 3*A*, RhoC mRNA expression increased in all of the cell lines on FTI treatment. A concordant increase in RhoC protein levels was also observed, as determined by Western blot analysis using a RhoC-specific antibody developed in our laboratory (33). The activity of RhoC was assessed using a GST-pulldown assay (34, 35). This assay utilizes a GST-fusion protein of a Rho-binding domain motif found in a variety of Rho-effector proteins. GTP-bound Rho is in its active state and can bind the Rho-binding domain (35). Using this assay, we found that in the SUM149 and HME-RhoC cell, the levels of GTP-bound RhoC were not affected by FTI treatment, which indicated that RhoC activation itself was unaffected by RhoB and RhoC accumulation. As expected from the mRNA and protein levels, activated RhoC was elevated in all of the FTI-treated cells, including the HME-β-gal control cells.

Because previous studies suggested a role for RhoB in reverting the malignant phenotype of Ras-transformed cells treated with FTI (42), we performed semiquantitative RT-PCR and Western blot analysis for RhoB. As shown in Fig. 3*B*, RhoB mRNA levels markedly increased 24 h after treatment with FTI L-744,832 in all of the cell lines tested. Furthermore, RhoB protein levels were also significantly increased.

These results support earlier observations that the accumulation of RhoB, likely RhoB-GG but not farnesylated RhoB, leads to a reversion of the malignant phenotype by FTI (30, 42, 43). The mechanism of FTI inhibition of the RhoC-induced phenotype appears to be independent of direct action of the FTI on geranylgeranylated RhoC. This is demonstrated by an accumulation of RhoC protein and no change in its activity.

Expression of RhoB-GG Recapitulates the FTI-mediated Effects on RhoC-overexpressing Breast Cells. In light of the previous experiments, we hypothesized that the accumulation of RhoB protein, specifically the RhoB-GG isoform that accumulates in FTI-treated cells, would be sufficient to revert the RhoC-induced phenotype. To test this hypothesis, we transiently transfected breast cells with a RhoB-GG construct and performed a focus formation and random motility assay. As controls, we also transfected cells with a geranylgeranyl-deficient mutant RhoB construct. Transfection efficiency was assessed by cotransfection with a *β-gal* reporter gene and found to be ~30–50% for the SUM149 and HME-RhoC cells. This assay could not be performed for the HME-β-gal controls since these cells already express the *β-gal* gene. However, RT-PCR using

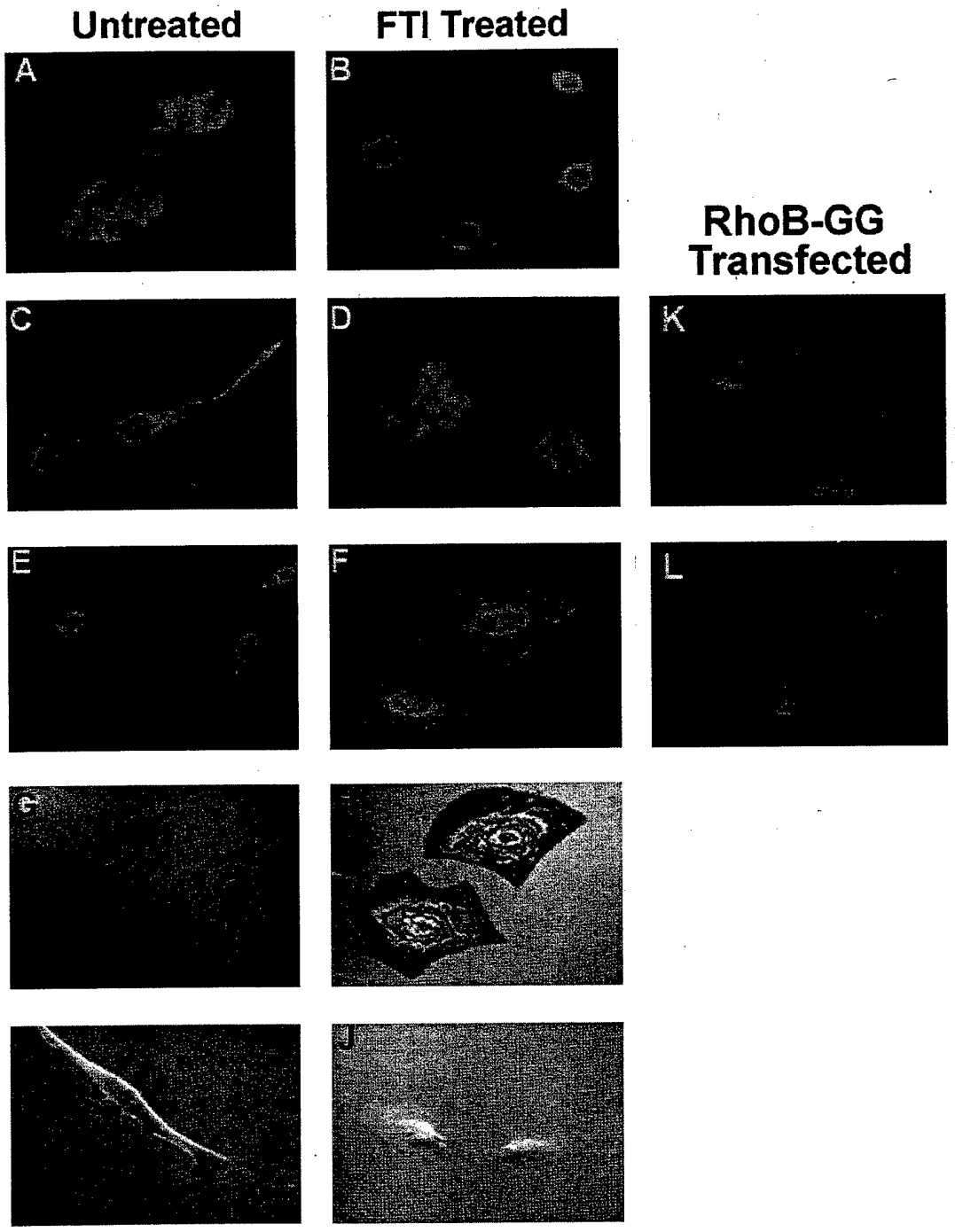

Fig. 2. Laser scanning confocal fluorescence microscopy (*A–F*) of untreated and L-744,832 treated cells after staining with a rhodamine-tagged phallotoxin that targets F-actin bundles. *A* and *B*, the HME-β-gal control cells untreated and FTI L-744,832-treated, respectively. *C* and *E* demonstrate a highly organized and polarized cytoskeleton in the SUM149 and HME-RhoC cells, respectively. *D* and *F* demonstrate dissociated actin bundles and decreased cellular polarity on FTI treatment. *G* and *H*, are laser scanning confocal microscopy images, and *I* and *J* are scanning electron microscopy images of untreated and FTI-treated HME-RhoC cells, respectively, showing the changes in cell morphology. *K* and *L* are RhoB-GG-transfected HME-RhoC and SUM149 cells, respectively.

vector-specific primers demonstrated mRNA expression in all of the cell lines tested.

Table 1 shows the results of a focus formation assay for the transfectants. The SUM149 and HME-RhoC cells, transiently transfected with RhoB-GG, formed significantly fewer foci than did the RhoB-GG-deficient mutant transfectants or the nontransfected cells. The morphology of the RhoB-GG-transfected cells were similar to their FTI-treated counter-

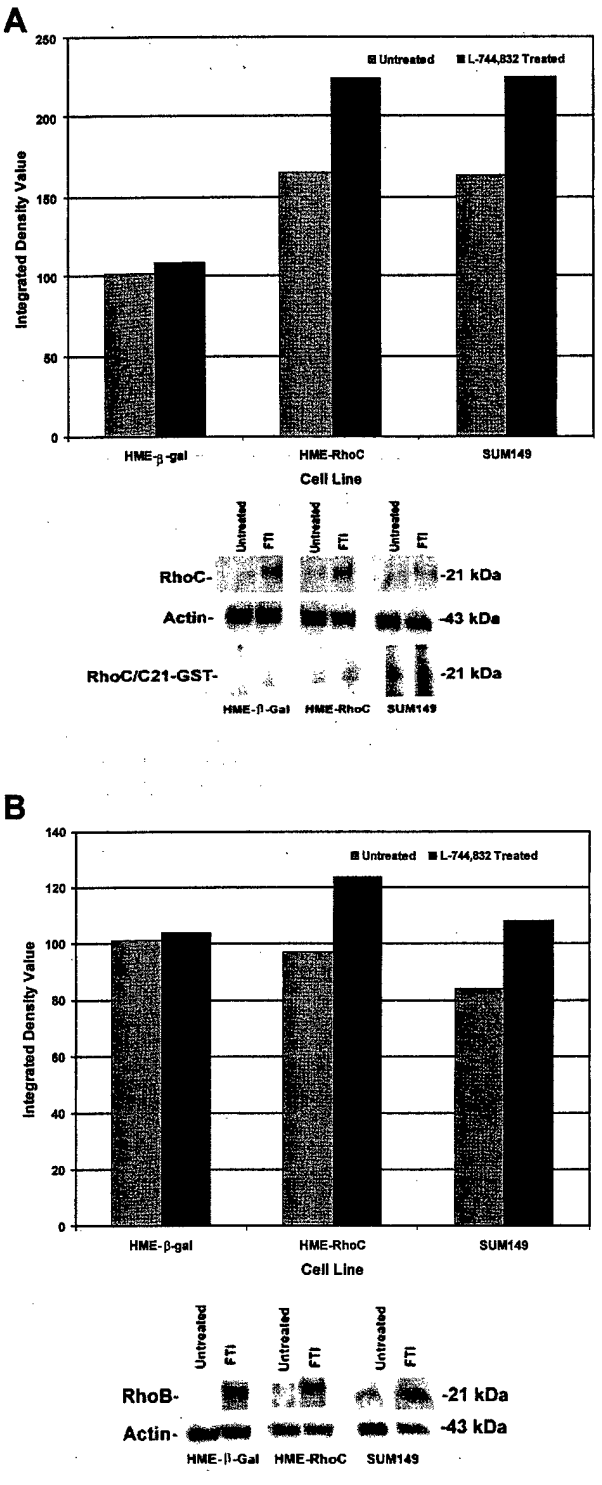

Fig. 3. Results of semiquantitative RT-PCR and Western blot analysis for RhoC GTPase (*A*) and RhoB GTPase (*B*). Densitometric comparison of the PCR products with a GAPDH internal standard demonstrated a modest increase in RhoC mRNA expression in all of the cell lines treated with FTI L-744,832 as compared with the untreated cell line. Similarly, an increase in RhoB mRNA expression was observed only for the HME-RhoC and SUM149 cell lines (*B*). A concurrent increase in RhoC protein levels was observed for the FTI-treated cell lines (*A*). This was accompanied by a slight increase in RhoC activity as determined by a C21-GST pulldown assay. As shown in *B*, RhoB protein levels increased in FTI-treated cells. *kDa, M*, in thousands.

Table 1 Mean focus formation and SD after transient transfection with RhoB-GG, a geranylgeranyl-deficient RhoB (RhoB-A3), and vector alone

The number of foci were assessed in triplicate 35-mm plates. The data presented are from a seeding density of 100 cells/plate. However, similar trends were seen when cells were seeded at 1000 or 500 cells/plate. Focus formation was dramatically reduced in the RhoC-overexpressing HME-RhoC and SUM149 cells by expression of geranylgeranylated RhoB, but not an unprenylated mutant (RhoB-A3). Although a trend was noted, statistical analysis by a Kruskal-Wallis test did not demonstrate a significant difference between the groups.

Cell line	Vector alone	RhoB-GG	RhoB-A3
HME-β-gal	48.5 ± 5.5	24.5 ± 1	36.5 ± 2.5
HME-RhoC	85 ± 3	23.5 ± 1	69 ± 6.5
SUM149	19.5 ± 1.5	5 ± 0.5	20.5 ± 1
MCF10AT c1	53.5 ± 0.5	25.5 ± 6	46 ± 1.5

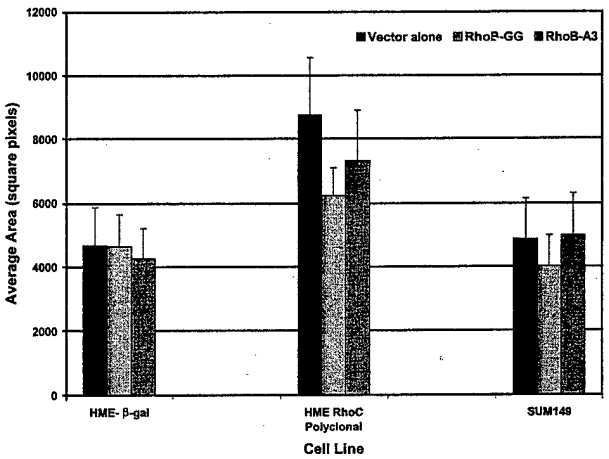

Fig. 4. Random motility assay of HME-β gal, HME-RhoC, and SUM149 cells transfected with either vector alone, RhoB-GG, or a geranylgeranyl mutant RhoB-A3. Although cell motility was not significantly altered, a trend was seen in the HME-RhoC and SUM149 cells transfected with RhoB-GG that resembled FTI treatment.

parts (Fig. 2, *K–L*). Similarly, the RhoB-GG transfectants were less motile when tested in the colloidal-gold assay (Fig. 4). Although statistical significance was not reached for the transfectants, the trends indicate that expression of RhoB-GG leads to decreased motility in RhoC-overexpressing breast cells. These data provide evidence for a role for RhoB-GG as a mechanism for inhibiting or reverting the RhoC-induced phenotype in these cells.

Discussion

The observation that H-Ras was inactive and not localized to its specific membrane compartment after FTI treatment led to the idea that these inhibitors could be used therapeutically (44–48). Like Ras, the Rho GTPases are posttranslationally modified to locate them to their distinct cellular compartment, so that they can carry out their specific function (17–19). Each Rho protein contains a COOH-terminal CAAX domain that determines prenylation and polybasic residues in the hypervariable domain, upstream of the CAAX domain, which dictate proper membrane localization (49).

Membrane localization and trafficking of the Rho GTPases are complex phenomena. Rho proteins are, in their inactive state, localized in the cytosol, sequestered there by specific RhoGD (25). On activation, the GTP-bound protein is prenylated and transported to its specific membrane compartment. The type of prenylation is dependent on the Rho protein (50–53). RhoC GTPase is geranylgeranylated, whereas RhoB is both geranylgeranylated and farnesylated (17–19, 50, 51).

It has been demonstrated that *in vitro*, Rho GTPases can self-aggregate (54). Cdc42 and Rac2 homodimer formation has been implicated in the negative regulation of the activity of those proteins (55). RhoB and RhoC GTPase have been found to exist, not as homodimers, but as either monomeric or oligomeric complexes. In addition, RhoC has been shown to have an arginine finger motif COOH-terminal to the CAAX domain, which imparts self-activated GTPase regulatory function (54). Specifically, GTP-bound RhoC, when in complex with itself, can self-convert to RhoC-GDP. In contrast, RhoB does not contain this arginine domain and, therefore, does not have intrinsic GAP activity. It is yet unknown whether RhoB and RhoC can form heterodimers either *in vitro* or *in vivo*. Because FTI cannot directly block geranylgeranyl RhoC function, one possible explanation for FTI suppression of RhoC function is that accumulation of RhoB-GG leads to the oligomerization of RhoC, which leads to increased intrinsic GAP activity and GTPase deactivation.

The promise of FTIs as a potent therapeutic reagent has been supported by *in vivo* studies. Mammary tumors that develop in K-*Ras* transgenic mice can be growth inhibited by FTI treatment (56). Lebowitz *et al.* (30) and Prendergast *et al.* (57–59) have provided have evidence that FTI suppression of Ras transformation was accomplished by interfering with Rho activity, because Rho was shown to be critical in Ras-induced transformation. Subsequent experiments demonstrated that a shift from farnesylated RhoB GTPase to RhoB-GG occurred on FTI treatment (30, 31, 60, 61). The shift in the specific forms of prenylated RhoB is accompanied by the accumulation and mislocalization of RhoB-GG, which is normally a short-lived protein (with a half-life of 2–4 h in cells; Refs. 30, 62). Our present data support these observations. In this study, we observed increased expression and accumulation of RhoB, presumably RhoB-GG, on treatment with FTI L-744,832. We also demonstrated that transient transfection of RhoC-overexpressing breast cells with RhoB-GG, recapitulated the effects of FTI treatment, inhibiting focus formation and random motility, whereas transfection with the RhoB GG-deficient mutant failed to mimic FTI effects.

In Ras-transformed cells, the effects of RhoB-GG may be attributable to a "gain-of-function" and relocalization of the protein (42, 43, 59, 63). Normally, RhoB GTPase is involved in vesicular and receptor trafficking (64). However, after FTI treatment, the inhibition of farnesylated RhoB and the accumulation of RhoB-GG, may lead to altered functions. The biosynthesis of geranylgeranyl PP_i is the next step after the synthesis of farnesyl PP_i in the acetyl-CoA pathway of cholesterol synthesis (reviewed by Cohen *et al.*; Ref. 48). Therefore, FTI treatment may provide more substrate for the geranylgeranyl PP_i synthase to produce geranylgeranyl PP_i and, ultimately, functionally geranylgeranylated Rho. Furthermore, several investigators suggest that a membrane receptor may exist that binds to the Rho prenyl-group, thereby helping to specifically localize it to a membrane compartment (51, 65–67). In this scenario, the accumulation of RhoB-GG may compete with RhoC, displacing it and possibly preventing it from interacting with downstream effector molecules.

In our experiments, we demonstrate that on FTI-treatment, RhoC levels also increase; however, the ratio of RhoB:RhoC remains increased over pretreatment levels. Furthermore, we speculate that RhoC may be accumulating in the cytoplasm, or, if it is reaching the inner membrane, its effect on the cell is attenuated by RhoB-GG. These ideas have yet to be tested. As demonstrated by labeling the actin cytoskeleton with a rhodamine-labeled phallotoxin, the polarized actin bundles associated with the motile RhoC cells are nearly lost on FTI treatment. Although focal adhesions are visible in both treated and untreated cells, they are located exclusively around the outside edges of the FTI-treated cells. Both laser confocal and scanning electron microscopy demonstrate that, as observed previously (57), the cells have lost their polarity and are flattened.

In contrast with Ras-transformed cells, the growth rate of the RhoC-overexpressing cells was only slightly affected by FTI treatment. Cell viability was also unaffected. Again, this is in contrast to previous experiments, which demonstrated that a combination of FTI and LY294002 (an inhibitor of P13K) led to increased apoptosis in Ras-transformed cells (31) but not in RhoC-overexpressing cells.

Taken together, these data suggest that FTIs may prove a potent novel therapeutic agent against tumors that overexpress RhoC GTPase. This is the first report of FTI inhibition of the cancer phenotype induced specifically by overexpression of RhoC GTPase. The mechanism of FTI action in RhoC-overexpressing IBC and HME transfectants may be similar to that previously described for Ras-transformed cells, namely, that the effect is mediated by the accumulation of RhoB-GG. However, as described above, there are notable differences in how FTI treatment affects cell growth in Ras-transformed *versus* RhoC-overexpressing cells. In addition to IBC, it has been demonstrated that aggressive noninflammatory, metastatic breast cancers, advanced pancreatic cancer, and metastatic melanoma overexpress RhoC GTPase, and this event significantly contributes to their clinical behavior; therefore, FTI treatment may be effective against these aggressive cancers (33, 68, 69). Our data support testing whether FTI treatment is efficacious in these aggressive RhoC-driven malignancies.

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Role for RhoB and PRK in the suppression of epithelial cell transformation by farnesyltransferase inhibitors

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Abstract

Recent genetic investigations have established that RhoB gain-of-function is sufficient to mediate the antitransforming effects of farnesyltransferase inhibitors (FTIs) in H-Ras-transformed fibroblast systems. In this study, we addressed the breadth and mechanism of RhoB action in epithelial cells transformed by oncoproteins which are themselves insensitive to FTI inactivation. Rat intestinal epithelial cells (RIE cells) transformed by activated K-Ras or Rac1 were highly sensitive to FTI-induced actin reorganization and growth inhibition, despite the inability to FTI to block prenylation of either K-Ras or Rac1. Ectopic expression of the geranylgeranylated RhoB isoform elicited in cells by FTI treatment phenocopied these effects. Analysis of RhoB effector domain mutants pointed to a role for PRK, a Rho effector kinase implicated in the physiological function of RhoB in intracellular receptor trafficking, and these findings were supported further by experiments in a fibroblast system. We propose that FTIs recruit the antioncogenic RhoB protein in the guise of RhoB-GG to interfere with signaling by prooncogenic Rho proteins, possibly by sequestering common exchange factors or effectors such as PRK that are important for cell transformation.

Introduction

Farnesyltransferase inhibitors (FTIs) are a class of signal transduction inhibitors in cancer clinical trials that are providing a useful new tool to study neoplastic pathophysiology. FTIs were designed to attack Ras oncoproteins, the function of which depends upon posttranslational modification by farnesyl isoprenoid. However, it has become increasingly clear and accepted that the drugs' antineoplastic properties can be traced to a large degree to an alteration in the prenylation and function of proteins other than Ras (Adjei, 2001; Cox, 2001; Prendergast, 2000a; Prendergast, 2001; Prendergast & Oliff, 2000; Rowinsky et al., 1999; Sebti & Hamilton, 2000). One candidate target that has emerged from studies of the cellular response to FTI is RhoB. Investigations of this endosomal Rho protein have revealed an antioncogenic function that is recruited in cells by FTI through a gain-of-function mechanism (Prendergast, 2000a; Prendergast, 2001). Genetic investigations have established that RhoB is sufficient and/or necessary to mediate many facets of the FTI response displayed in mouse and rodent model systems or in human cancer cells (Du et al., 1999a; Du & Prendergast, 1999; Liu et al., 2000). These findings have been extended recently by the identification of RhoB as a negative modifier function in cancer cells that is required for the antineoplastic response to DNA damaging agents and paclitaxel (Liu et al., 2001a; Liu et al., 2001b). Thus, the use of FTIs to probe neoplastic pathophysiology has yielded two new concepts: the concept that Rho signaling affects chemotherapeutic responses, and the concept that cancer chemotherapeutics must recruit negative modifier functions to effectively target neoplastic cells.

Important remaining issues regarding RhoB in the FTI response include the following. First, the main evidence for this link has been obtained in cells transformed by H-Ras, which is a relatively less important human oncogene than K-Ras. Is RhoB involved in the FTI response in K-ras-transformed cells? This question bears on the long-standing issue of how FTI manages to suppress K-ras transformation without blocking K-ras prenylation (due to the ability of geranylgeranyltransferase-I [GGT-I] to prenylate and maintain the function of K-ras in FTI-treated cells (Lerner et al., 1997; Rowell et al., 1997; Whyte et al., 1997). Second, what effector signaling pathways does RhoB use to mediate its antiproliferative effects in transformed cells? Work from Mellor and colleagues has defined a function for RhoB in intracellular receptor trafficking (Gampel et al., 1999), which depends upon interactions with the Rho effector kinase PRK (Flynn et al., 2000; Mellor et al., 1998), but it is not known whether this pathway is involved in the antiproliferative actions of RhoB in transformed cells. This question also bears on the relationship between RhoB and its prooncogenic relatives, the RhoA and RhoC proteins, which can also interact with PRK as well as other Rho effector proteins (due to the identical effector domains on the RhoA/B/C proteins). Prooncogenic Rho signals are required for transformation by Ras (Khosravi-Far et al., 1995; Qiu et al., 1995). Marshall and colleagues have proposed that this requirement is based on the negation of cell cycle kinase inhibitors (CDKIs) that are induced by oncogenic Ras (Olson et al., 1998). Because of the identity of the effector domains in the RhoA/B/C proteins, the simplest way to interpret the antagonistic effects of RhoB on transformation is to propose that it antagonizes signaling from the prooncogenic RhoA/C proteins (Prendergast, 2001), perhaps by sequestering common Rho effector molecules such as PRK kinases. A strong prediction of this model is that FTI should block transformation by prooncogenic Rho proteins that are geranylgeranylated. Work in this study and elsewhere

(van Golen et al., 2002) offers evidence to support this model, which may promote insights into RhoB function and its role in the response to FTI and other chemotherapeutics.

Materials and Methods

Cell Culture. All cells were cultured in Dulbecco modified Eagle medium (DMEM; Life technologies) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Rat intestinal epithelial (RIE) cells were transformed with an activated K-Ras-4B(G12V) cDNA expressed from a neo-tagged pZIP retroviral vector or with an activated Rac1(Q61L) cDNA expressed from a neo-tagged pcDNA3 vector (Invitrogen). Cells were passaged 48 hr after transfection in media containing 500 μg/ml G418 and transformed colonies were ring cloned and expanded as clonal cell lines. Transgene expression in transformed cell lines was confirmed by Western analysis. Multiple clones tested for FTI responsiveness yielded highly similar results; representative data from a single randomly chosen K-Ras or Rac1 clone is presented in the study. RIE/K-ras or RIE/Rac1 derivatives that expressed RhoB-GG or RhoB-GG effector mutants were generated by infection with recombinant retroviruses. For these experiments, a hemagglutinin antigen epitope (HA)-tagged RhoB-GG cDNA that had been described previously (Du et al., 1999a) was subcloned into the replication-incompetent retroviral vector MSCVpac that includes a puromycin selection marker (Hawley et al., 1994). Effector mutants generated in the HA-RhoB-GG cDNA were similarly subcloned into MSCVpac (oligonucleotide sequences used for plasmid constructions are available upon request). Recombinant MSCVpac viruses were packaged by standard methods in Phoenix cells. After infection cells were passaged in DMEM media containing 1 μg/ml puromycin and drug-resistant cell populations were pooled for

analysis. The mutant v-H-Ras-transformed Rat1 cell line Rat1/ras used in this study has been described previously (Du & Prendergast, 1999; Kohl et al., 1993). Rat1/ras cells were transfected with epitope-tagged wildtype or kinase-dead PRK2 expression vectors or with the pcDNA3 vector alone using the commercial CalPhos mammalian transfection kit, using the vendor's instructions (Clontech). Cells were passaged 48 hour after transfection in media containing 100 μg/ml zeocin, ring cloned, and expanded as cell lines. Transgene expression was confirmed in cell populations by Western analysis.

Western analysis. Cell extracts prepared in NP40 lysis buffer were analyzed by standard blotting methods using a commercial chemiluminescence kit to develop the blots (Pierce). K-RasV12 was detected with sc-30 mouse monoclonal antibody, Rac1 was detected with sc-217 rabbit antisera, and endogenous RhoB was detected with sc-180 rabbit antisera (Santa Cruz Biochemicals); HA-tagged RhoB transgene was detected with anti-HA mouse monoclonal antibody 12CA5 (Roche Biochemicals); PRK2 was detected with anti-PRK2 antibody (Transduction Laboratories); myc epitope-tagged kinase-dead PRK2 was detected with anti-myc monoclonal antibody 9E10; V5 epitope-tagged PRK2 transgene was detected using an anti-V5 antibody (Invitrogen).

Cell morphology. Cells were seeded in a 10 cm tissue culture dish and photographed 24 hr later at 100x magnification under the conditions noted. In FTI experiments, dimethyl sulfoxide (DMSO) was added as vehicle in control trials to no more than 0.1% (v/v) of culture media.

Cell proliferation and viability assays. Anchorage-dependent cell growth in monolayer cultures was measured by MTT [3- (4,5-dimethylthiazoly-2-yl)2,5-diphenyltetrazolium bromide] assay (Roche) according to manufacturer's protocol. Briefly, cells were seeded at 2000 cells per well in 96-well culture plates in quadruplicate. At appropriate times, 0.5 mg/ml MTT was added to each well and the 96-titer plate was incubated at 37^{0}C in a humidified incubator in which 5% CO_2 was maintained. After 4h 100 μl of solubilization buffer (10% SDS in 0.01 M HCl) was added to each well and incubated overnight in a humidified atmosphere before quantification. To monitor anchorage-independent cell proliferation, colony formation assays in soft agar culture were performed as described previously (Du et al., 1999a). Apoptosis was quantitated by flow cytometry using standard methods that have been described previously (Liu et al., 2001a; Liu et al., 2000). The proportion of apoptotic cells in a population was defined as the proportion of cells exhibiting sub-G1 phase DNA.

Actin immunofluroscence. Cells were seeded onto coverslips in six-well dishes and treated 24 hr with 10 μm FTI or an equivalent volume of vehicle. Cells were fixed and stained with fluorescein-phalloidin (Molecular Probes) and photographed using indirect immunofluorescence microscopy as described previously (Prendergast et al., 1994).

Transcription assay. A derivative of the SRE reporter construct 3D.ACAT that used luciferase instead of chloramphenicol acetyl transferase as the reporter gene was employed to probe the effects of various RhoB effector mutations on SRE activation. Transcription assays were performed essentially as described previously, using the ß-galactosidase reporter plasmid CMV-ß-gal to normalize for transfection efficiency (Lebowitz et al., 1997b).

Results

Intestinal epithelial cells transformed by K-Ras or Rac1 are highly susceptible to FTI treatment: association with elevation of RhoB-GG. RIE/K-ras cells were generated by transformation with the activated K-Ras mutant K-Ras4B-G12V. The response of these cells to farnesyltransferase inhibition was studied using the FTI L-744,832, a highly potent and specific compound that does not block the activity of cellular geranylgeranyltransferases (Kohl et al., 1995). Control experiments to examine the effects of FTI on untransformed RIE cells employed the matched vector cell line RIE/neo. FTI treatment rapidly elicited an elevation in the levels of the geranylgeranylated isoform of RhoB that is synthesized in drug-treated cells (RhoB-GG), consistent with previous observations (Lebowitz et al., 1997a). RhoB-GG induction was more pronounced in RIE/K-ras cells but in both cell lines its elevation was detectable as early as 4 hr after drug treatment, preceding any apparent cellular response (Fig. 1A). Within 12-16 hr a dramatic shift in the morphology of drug-treated RIE/K-Ras cells became apparent (Fig. 1B). The flattened and enlarged morphology observed was highly reminiscent of the phenotypic reversion displayed in H-Ras transformed fibroblasts, and unexpectedly, due to the reported 'resistance' of K-Ras-transformed fibroblasts to FTI treatment, within the same dose range. While less dramatic, the morphology of untransformed RIE/neo cells was altered similarly with the same kinetics and dose range (Fig. 1B). Following work in fibroblast systems, which links phenotypic reversion to a RhoB-dependent reorganization of the actin cytoskeleton (Du et al., 1999a; Liu et al., 2000), we examined the organization of F-actin in drug-treated RIE cells. As expected, K-Ras transformation was associated with a complete disruption of the stress fiber network that characterized untransformed RIE cells (Fig. 1C). Similar to what is seen in

fibroblast models (Prendergast et al., 1994), the morphological shift produced by FTI was associated with the reinstatement of a stress fiber network in RIE/K-ras cells and with the stimulation of a more robust stress fiber network in RIE/neo cells. RhoB-GG is sufficient and necessary to mediate FTI-induced actin reorganization in H-Ras-transformed fibroblasts (Du et al., 1999a; Liu et al., 2000); the induction of RhoB-GG by FTI in RIE cells was consistent with a similar role here.

The proliferation of RIE/K-ras cells were also highly sensitive to disruption by FTI treatment, despite the noted inability of FTI to block K-Ras prenylation and therefore function. The proliferation of RIE/K-ras cells in monolayer culture was virtually halted by FTI treatment (Fig. 2A). The IC50 for this effect was similar to from that required to block the proliferation of H-Ras-transformed fibroblasts (data not shown). By comparison, the division of RIE/neo cells was slowed, but not stopped, consistent with previous demonstrations that FTIs more effectively target the growth of transformed cells. Under anchorage-independent conditions, where RIE/K-ras but not RIE/neo cells were capable of cell division, a similar sensitivity of RIE/K-ras cells to growth suppression by FTI treatment was demonstrated (Fig. 2B). Consistent with observations in K-Ras-transformed NRK fibroblasts (Suzuki et al., 1998), RIE/K-ras cells were susceptible to FTI-induced apoptosis under low growth factor conditions (data not shown). The dose required to suppress colony formation or induce apoptosis under these different conditions (1-5 μM L-744,832) was in the same range as that determined in assays using H-Ras-transformed fibroblasts. These observations also contrasted with the reported 'resistance' of K-Ras-transformed fibroblasts to FTI treatment. We concluded that, despite the well-documented inability of FTI to block K-Ras prenylation, due to its alternate geranylgeranylation in FTI-

treated cells (Lerner et al., 1997; Rowell et al., 1997; Whyte et al., 1997), RIE/K-ras cells were fully susceptible to the antitransforming effects of FTI treatment.

To explicitly test whether FTI could bypass a geranylgeranylated oncoprotein to suppress cell transformation, we investigated the response of Rac1-transformed cells to FTI treatment. Rac1 is solely geranylgeranylated in cells so FTI L-744,832 can not affect the function of this oncoprotein. The activated mutant Rac1-Q61L was used to generate transformed RIE/rac1 cells. As before, FTI treatment rapidly elicited an elevation in the cellular levels of RhoB-GG within 4 hr of drug treatment, preceding any apparent cellular response (Fig. 3A). The phenotype of RIE/rac1 cells was comparatively flat, relative to RIE/neo control cells, pronounced membrane ruffles were apparent which gave the cells a hard-edged appearance. Within 12-16 hr of drug treatment, a shift in the morphological phenotype could be seen, with a loss of the pronounced membrane ruffles and a subtle flattening of cell shape (Fig. 3B). F-actin staining revealed the presence of both ruffles (blurred in the figure) and stress fibers in untreated RIE/rac1 cells (Fig. 3C). More pronounced pseudopods were also apparent. Interestingly, FTI treatment diminished the ruffles and pseudopods but had little overt effect on stress fiber organization (Fig. 3C). The proliferation of RIE/rac1 cells in monolayer culture was suppressed by FTI treatment, but under these conditions not as robust as produced in RIE/K-ras cells (Fig. 3D). However, under anchorage-independent conditions, growth suppression was more robust and comparable to that seen in RIE/K-ras cells (Fig. 3E). As before, the antiproliferative effects were achieved at dose ranges that were comparable to those required to block the anchorage-independent growth of H-Ras-transformed fibroblasts. We were unable to perform the apoptosis experiment, because RIE/rac1 cells underwent apoptosis when deprived of serum growth factors (data not shown).

Nevertheless, these observations indicated that RIE1/rac1 cells were susceptible to the antitransforming effects of FTI, despite its inability to block the geranylgeranylation and therefore the function of the Rac1 oncoprotein. We concluded that FTI could bypass geranylgeranylated 'driver' oncoproteins to elicit reversion and suppress proliferation in transformed cells.

RhoB-GG phenocopies the actin reorganization and growth suppression produced by farnesyltransferase inhibition in transformed intestinal epithelial cells. In H-Ras-transformed fibroblast model systems, the RhoB-GG elicited by FTI is sufficient and necessary to mediate actin reorganization and sufficient to suppress proliferation (Du et al., 1999a; Liu et al., 2000). The ability of FTI to elicit RhoB-GG in RIE cells prompted us to confirm whether ectopic RhoB-GG was sufficient to phenocopy the FTI response in these cells. RIE/K-ras or RIE/neo cell populations that stably expressed RhoB-GG or vector sequences were generated by infection with recombinant retroviruses (Fig. 4A). RhoB-GG expression caused a slight flattening of RIE/neo cells, increasing their size to a degree similar to FTI treatment (Fig. 4B, top panels). RhoB-GG also caused a flattening of RIE/K-ras cells, but only to a partial extent that did not fully mimic that achieved by FTI treatment (Fig. 4B, bottom panels). The partial effect seen might reflect a dose response; FTI induces endogenous RhoB-GG to much higher levels in RIE/K-ras cells than RIE/neo cells (Fig. 1A), so the dose of ectopic RhoB-GG might reach the dose needed to induce the full morphological shift in RIE/neo cells, but not in RIE/K-ras cells. Alternately, RhoB-GG might not be sufficient to phenocopy the morphological shift fully. The latter interpretation is perhaps more appealing, insofar as ectopic RhoB-GG could phenocopy the actin stress fiber induction to a more similar extent in both RIE/neo and RIE/K-ras cells (Fig.

4C). This result argues that the induction of endogenous RhoB-GG by FTI is sufficient to mediate actin reorganization. Ectopic RhoB-GG also effectively phenocopied the growth suppression of RIE/K-ras cells by FTI, both in monolayer culture (data not shown) and under anchorage-independent conditions (Fig. 4D). Similar antiproliferative effects of RhoB-GG were observed under the same conditions in RIE/rac1 cells (data not shown). We concluded that the induction of RhoB-GG by FTI was a sufficient cause to mediate actin stress fiber formation and growth inhibition.

Evidence of a role for the RhoB effector kinase PRK in the antiproliferative effects of farnesyltransferase inhibition in transformed cells. The results above suggest that RhoB-GG indirectly interferes with cell transformation by K-Ras or Rac1, consistent with other evidence that RhoB has antioncogenic role in cells (Liu et al., 2001a; Liu et al., 2001b). To probe the basis for its activity, we investigated the effect of various effector domain mutations in RhoB-GG. RhoB is ~90% identical to RhoA in its primary structure, and in particular, the effector domains of these small GTPases are identical. Thus, effector molecules that have been defined through interaction with RhoA have in every case tested also interacted with RhoB, as would be expected on the basis of structural considerations. In a retroviral vector, we introduced into RhoB-GG six different effector domain mutations that have been shown in RhoA to affect interactions with various Rho effector molecules to variable degrees (Table I). RIE/K-ras cell populations that expressed these mutant derivatives were then examined in the assays for actin organization and anchorage-independent growth used above. Since the response of cells to FTI and RhoB is influenced by serum growth factors (Liu et al., 2000), we also tested the effector mutants for their ability to activate transcription of the serum response element (SRE), a

transcriptional target of RhoB (Lebowitz et al., 1997b). The results of these experiments are summarized in Table I and shown in Figures 5 and 6.

The set of effector domain mutants tested discriminated between possible mechanisms of growth suppression and SRE activation, but not actin reorganization. All the effector mutants were able to induce actin stress fibers to a considerable degree in RIE/K-ras cells (Fig. 5A). These observations included the T37Y mutant which binds weakly to most Rho effector molecules (Sahai et al., 1998). This result argued that interactions with several different effectors could trigger actin reorganization, any one of which may be sufficient. In contrast to this result, two mutants clearly discriminated interactions with the Rho effector kinase PRK as important for suppression of anchorage-dependent growth and SRE activation (Fig. 5B and Fig. 6). The patterns of activity for F39L and E40T, mutants which retained the ability to bind PRK, linked productive interactions with RhoB-GG to its ability to suppress proliferation and activate transcription (Table I). This connection was satisfying, insofar as the results were highly consistent with the evidence that PRK interaction is crucial for the physiological function of RhoB in the intracellular trafficking of the EGF receptor and perhaps other receptors (Gampel et al., 1999; Mellor et al., 1998).

To further probe the relationship of PRK to the FTI response, we expressed it in Rat1/ras fibroblasts, an H-Ras-transformed cell system that has been widely used for FTI studies (Du et al., 1999a; Du et al., 1999b; Kohl et al., 1993; Lebowitz et al., 1995; Lebowitz et al., 1997c; Prendergast et al., 1994). For these experiments we used PRK2 since this PRK isoform is expressed in Rat1/ras cells; both PRK1 and PRK2 are recruited similarly by RhoB to endosomal

vesicles (Gampel et al., 1999; Mellor et al., 1998; H. Mellor, pers. comm..). To probe possible effects of the PRK kinase domain or membrane recruitment, we also expressed a kinase-dead mutant (PRK2-KD) or an N-myristoylated derivative of PRK2 in Rat1/ras cells. Several clones analyzed for each Rat1/ras derivative acted similarly; results from representative clones are presented here (Fig. 7A). Interestingly, ectopic PRK2 relieved the requirement for serum deprival for FTI to induce apoptosis in Rat1/ras cells. Under control conditions (i.e. monolayer culture in media containing 10% fetal bovine serum), FTI treatment induces reversion and growth inhibition in Rat1/ras fibroblasts, whereas if serum growth factors are deprived, FTI treatment triggers an apoptotic program (Du et al., 1999c). In contrast to vector control cells, which acted like the parental cells, PRK2-expressing cells underwent apoptosis in serum-containing media as evidenced in phase micrographs or flow cytometry (Figs. 7B and 7C). The kinase activity of PRK2 was implicated, because cells expressing the kinase-dead PRK-KD mutant did not recapitulate the effect, instead retaining the requirement of serum deprivation for FTI-induced apoptosis. Myr-PRK2-expressing cells were resistant to FTI-induced apoptosis. Myristylation relieves the requirement for Rho to mediate membrane recruitment. Therefore, the ability of myr-PRK2 to block apoptosis suggested that the proapoptotic effects of PRK2 in the FTI response were linked not only to its kinase activity but also to its localization and/or movement in cells. This observation was notable in light of the evidence that FTI alters the localization as well as the level of RhoB-GG in cells, and that both changes might be needed for the FTI response (Adamson et al., 1992; Du et al., 1999a; Lebowitz et al., 1995). Taken together with the RIE results, these experiments supported a role for PRK activity and localization in the RhoB-mediated facets of the FTI response.

Discussion

The results of this study offer direct evidence that RhoB gain-of-function is a sufficient cause for FTI to bypass the action of farnesyl-independent oncoproteins in suppressing cell transformation. The work strengthens the connection between RhoB and the FTI response by confirming its relevance in cells of epithelial origin, where actin-dependent polarity signals that impact transformation and proliferation processes likely occur through distinct mechanisms that are irrelevant in fibroblasts. The results also support a role for interactions with the Rho effector protein PRK in mediating RhoB-dependent facets of the FTI response. PRK is a critical effector for the physiological function of RhoB in intracellular receptor trafficking, and our observations are consistent with a link between this function and the antiproliferative effects of RhoB.

K-ras-transformed RIE cells were highly susceptible to FTI treatment, lacking the relative 'resistance' observed by some laboratories in K-ras-transformed fibroblasts (compared to H-Ras-transformed fibroblasts). Although this resistance has been ascribed to the relatively higher avidity of K-Ras for FT, compared to H-Ras, our observations suggest that the 'resistance' phenomenon is a cell type-specific property unrelated to the avidity of different Ras proteins for FT. Indeed, since the oncogenic activity of K-Ras remains intact when the protein is geranylgeranylated, as it is in FTI-treated cells, the ability of FTI to suppress K-ras transformation is more readily explained by effects on non-Ras targets. Our observations rule in RhoB gain-of-function as a sufficient cause for explaining the suppression of K-ras transformation by FTI.

Another illustration that FTI can bypass geranylgeranylated oncoproteins to suppress transformation was provided by the demonstration that RIE/rac cells were susceptible to FTI. Rac1 is solely geranylgeranylated in cells, so FTI must act by indirectly to blocking transformation; the induction of RhoB-GG and its ability to phenocopy FTI effects as in RIE/K-ras cells is consistent with a role for RhoB in the drug response. These observations have been corroborated and extended still further by the demonstration that FTI treatment both induces RhoB and suppresses RhoC-dependent transformation of human mammary epithelial cells (van Golen et al., 2002). This result has clinical implications, because RhoC overexpression occurs in >90% of inflammatory breast cancers and ectopic RhoC recapitulates phenotype of these cancers (van Golen et al., 1999; van Golen et al., 2000a; van Golen et al., 2000b). FTI was shown to inhibit the anchorage-independent growth, motility, and invasion of RhoC-transformed cells, and ectopic RhoB was sufficient to phenocopy these effects (van Golen et al., 2002). Like Rac1, RhoC is solely geranylgeranylated in cells, so these observations strongly reinforce the notion that FTIs interfere with prooncogenic Rho signals by eliciting RhoB-GG.

We obtained genetic evidence supporting a role for PRK in RhoB-dependent facets of the FTI response. Effector mutations that abolished PRK interactions effectively limited the ability of RhoB-GG to suppress anchorage-independent growth or to activate SRE transcription. One implication is that antiproliferative effects of RhoB may involve downstream transcriptional events, a possibility supported by evidence that FTI-induced reversion in Rat1/ras cells requires derepression of the collagen Iα2 gene (Du et al., 1999b). Another implication is that the antiproliferative and transcriptional effects of RhoB are derivative of the physiological function of RhoB in intracellular receptor trafficking, which also depends upon PRK interaction (Flynn et

al., 2000; Gampel et al., 1999; Mellor et al., 1998). Experiments in a fibroblast model system supported a role for PRK in the FTI response. Elevation of PRK relieved the serum deprival requirement for FTI-induced apoptosis, in a manner dependent upon the integrity of the PRK kinase domain, whereas elevation of myristylated PRK (which does not require Rho interaction for membrane localization) blocked FTI-induced apoptosis. The latter observation argued that PRK localization is important: the ability of PRK to block FTI-induced apoptosis when PRK was localized to membranes in a Rho-independent fashion argued that movement away from membrane-associated sites was integral to its participation in the FTI response. This interpretation of the results is consistent with the evidence that FTI both elevates and relocalizes RhoB in cells (Lebowitz et al., 1995). Although PRK2 has been reported to be cleaved and activated by caspases during apoptosis (Cryns et al., 1997; Takahashi et al., 1998), we did not observe this event. A link between PRK and FTI-induced apoptosis is potentially interesting based on reports of the ability of PRK to complex with RhoB and the Akt-regulatory enzyme PDK1 in certain epithelial and endothelial cells (Balendran et al., 1999; Flynn et al., 2000; L. Benjamin, pers. comm..), the potential of cleaved PRK to modulate Akt activity (Koh et al., 2000), and the ability of RhoB-GG to phenocopy the inhibition of Akt activity by FTI in epithelial and endothelial cells (Liu & Prendergast, 2000; L. Benjamin, pers. comm.). Further investigations may shed additional light on the signaling mechanisms that relate PRK to the RhoB-dependent aspects of the FTI response.

The most informative mutations in the RhoB effector domain that were tested in this study were the F39L and F39V mutations, which differentially distinguish PRK interaction (Table I). RhoB-GG-mediated growth suppression was maintained by F39L mutation, which

supports PRK interactions, but was abolished by F39V mutation, which abolishes PRK interaction. Notably, the same mutations made in the prooncogenic RhoA protein abolish its cell transforming activity with Raf N-terminal truncates (Sahai et al., 1998; Zohar et al., 1998). Thus, F39L mutation suppresses the transforming activity of RhoA, whereas F39V supports this activity. Taken together, these results suggest that the antioncogenic function of RhoB is based on its competition with RhoA for PRK binding, insofar as RhoB must bind PRK to suppress transformation and RhoA must bind PRK to drive it. The induction and relocalization of RhoB-GG caused by FTI treatment may sequester PRK, competitively inhibiting its ability to bind to RhoA and to mediate prooncogenic signals. This model is consistent with the Rat1/ras results which suggested that PRK localization or movement is important to the FTI response. It is also consistent with the evidence that FTI elicits a relocalization of RhoB (i.e. RhoB-GG) in drug-treated cells (Lebowitz et al., 1995; H. Mellor, pers. comm.). As mentioned above, Ras transformation requires prooncogenic Rho signals; therefore, by eliciting RhoB-GG FTI may interfere with prooncogenic Rho signals to block Ras transformation (see Fig. 9). This model can also offer an explanation for how RhoB-GG mediates the strongly antagonistic effects of FTI on RhoC transformation (van Golen et al., 2002), through the ability of RhoB-GG to compete with RhoC for PRK interactions. The notion that RhoB antioncogenic activity is based upon competition for effectors or nucleotide exchange factors (GEFs) of prooncogenic Rho proteins is consistent with structural considerations and offers considerable explanative and predictive power. The utility of FTI to interfere with Rho signaling through these mechanisms may find clinical utility in novel disease settings that involve Rho, including inflammatory breast cancer, melanoma, and age-associated blindness as we have suggested (Prendergast, 2000b; Prendergast & Rane, 2001).

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Tables

Table I. PRK interactions correlate with the ability of RhoB-GG to inhibit transformed cell growth and to activate SRE-dependent transcription. The actin, growth inhibition, and SRE activation results are summarized from Figures 5 an 6. The Rho effector binding data is adapted from Sahai et al. (Sahai et al., 1998); effector domain sequences in RhoA and RhoB are identical.

				Binding of Rho effectors to Rho mutants						
RhoB mutant	**Actin**	**Growth Inhibition**	**SRF activation**	PRK	ROCK	Rhophilin	Kinectin	Citron	mDia	mNET
Wild-type RhoB-GG	++	++	++	++	++	++	++	++	++	++
T37Y	++	–	–	–	–	–	+/–	–	–	–
F39A	++	–	–	–	–	–	–	–	++	–
F39L	+	++	++	+	+	+	–	–	++	+
F39V	++	–	–	–	++	–	–	–	++	++
E40T	++	+	++	+	++	++	–	++	++	+
Y42C	++	–	–	–	++	+	+	++	++	+

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Figure Legends

Figure 1. Induction of RhoB-GG accompanies phenotypic reversion of K-Ras-transformed RIE cells by FTI. (A.) Western analysis. Cells were treated for the times indicated with 10 μM FTI L-744,832 before harvest and extract preparation. Blots were probed with a rabbit polyclonal anti-RhoB antisera. (B.) Morphology. Cells seeded into monolayer culture were photographed 24 hr after treatment with 10 μM FTI or vehicle control. (C.) Actin organization. Cells seeded onto coverslips were treated as above, fixed, and processed for F-actin staining using FITC-conjugated phalloidin.

Fig. 2. K-ras-transformed RIE cells are highly sensitive to growth suppression and apoptosis by FTI. (A.) Anchorage-dependent proliferation. Cell proliferation in monolayer culture was monitored by SRB assay. (B.) Anchorage-independent growth. Colony formation in soft agar culture was documented by photomicrography 12 days after seeding 10e4 cells per well in 6 well dishes. (C.) Apoptosis assay. The percentage of sub-G1 phase cells as determined by flow cytometry was used to quantify the extent of apoptosis in cell populations treated 24 hr in monolayer culture with 10 μM FTI. The percentage in each panel notes the proportion of the population that scored in the sub-G1 phase.

Figure 3. Induction of RhoB-GG accompanies actin reorganization and growth suppression of Rac-transformed RIE cells by FTI. (A.) Western analysis. Cells were treated 4h with 10 μM FTI L-744,832 before harvest and extract preparation. Blots were

probed with anti-RhoB antisera as before. (B.) Morphology and actin organization. Cells seeded into monolayer culture were photographed 24 hr after treatment with 10 μM FTI or vehicle control. For actin staining, cells seeded onto coverslips were treated and processed as before using FITC-conjugated phalloidin. (C.) Anchorage-dependent proliferation. Cell proliferation in monolayer culture was monitored by SRB assay as before. (D.) Anchorage-independent growth. Colony formation in soft agar culture was documented by photomicrography 14 days after seeding 10e4 cells per well in 6 well dishes. (E.) Apoptosis assay. The extent of apoptosis in cell populations treated 24 hr in monolayer culture with 10 μM FTI was determined as above. Serum deprivation by itself triggered apoptosis in RIE/rac cells and FTI accentuated this response slightly. The percentage in each panel notes the proportion of the population that scored in the sub-G1 phase.

Figure 4. RhoB-GG phenocopies actin reorganization and growth suppression by FTI. (A.) Western analysis. Expression of ectopic HA-RhoB-GG in extracts from cells that were stably infected by the puromycin-resistant retroviral vectors indicated was confirmed by blotting with anti-HA antibody, which recognizes the hemagglutinin epitope tag on the transgene. (B.) Morphology. Cells in monolayer culture were photographed 24 hr after seeding in the presence or absence of 10 μM FTI as indicated. (C.) Actin organization. Cells seeded onto coverslips were treated as above and processed as before for actin staining with FITC-conjugated phalloidin. (C.) Anchorage-independent growth. Colony formation in soft agar culture was documented by

photomicrography 14 days after seeding 10e4 cells per well in 6 well dishes. Vector cells were treated where indicated with 10 μM FTI as before.

Figure 5. Effects of effector domain mutations on growth suppression and actin organization by RhoB-GG. (A.) RIE/K-ras cell populations that were derived from infections with retroviral vectors expressing no insert (vector), RhoB-GG, or the RhoB-GG effector domain mutants indicatd were seeded into soft agar culture. Colony formation was documented by photomicrography 12 days after seeding. (B.) The same RIE/K-ras cell populations derived above were seeded onto coverslips and processed the next day for actin staining with FITC-conjugated phalloidin as before.

Figure 6. Effects of effector domain mutations on SRE-dependent transcriptional activation by RhoB-GG. Normalized luciferase activity was determined in cell extracts prepared 48 hr after transfection of NIH3T3 cells with an SRE-luciferase reporter plasmid and vectors expressing no insert (vector), RhoB-GG, or the RhoB-GG effector domain mutant indicated.

Figure 7. Impact of PRK activity and membrane association on the FTI response of H-Ras-transformed fibroblasts. (A.) Western analysis. Expression of PRK2, PRK2-KD (myc epitope-tagged kinase-dead PRK2 mutant), or myr-PRK2 (V5 epitope-tagged N-myrisylated PRK) in extracts prepared from Rat1/ras cell derivatives was documented on Western blots probed with anti-PRK2, anti-myc 9E10, or anti-V5 antibodies, respectively. (B) Morphology. Rat1/ras cell clones indicated were incubated 8 hr after

seeding at 10e6 cells per 10 cm dish in DMEM containing 10% FBS or 0.1% FBS. The next day 10 μM FTI or an equivalent volume of DMSO vehicle (Control) was added and 24 hr later cell morphology was documented by photomicrography. (C) Flow cytometry. Rat1/ras cell clones indicated were treated as above and harvested for flow cytometry 24 hr after FTI treatment.

A)

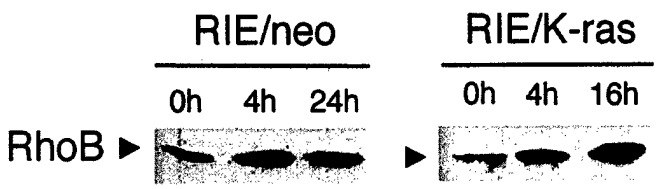
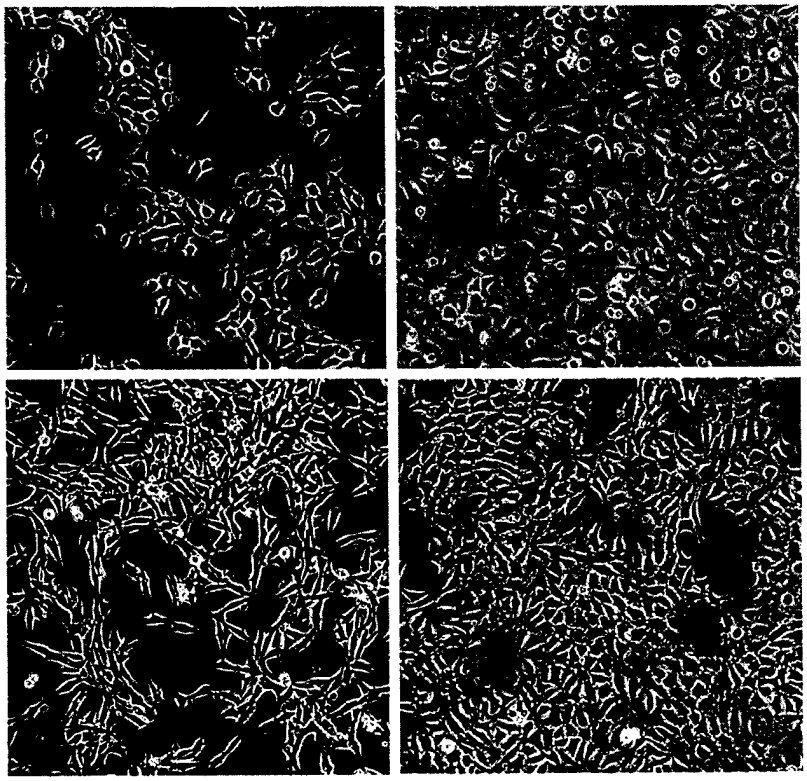
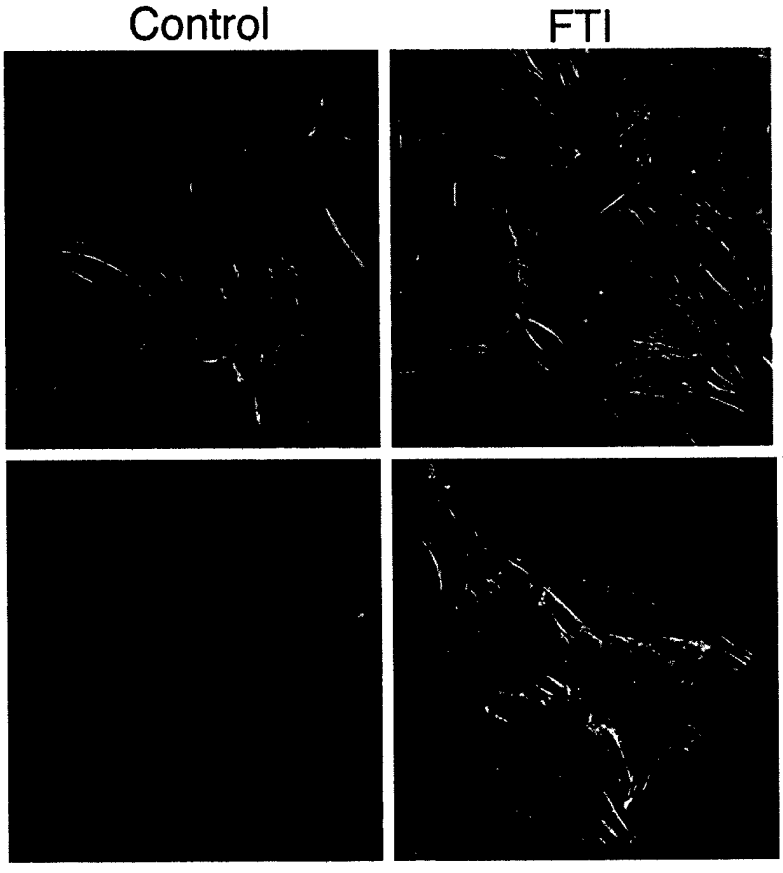

RIE/neo
RIE/K-ras
0h 4h 24h
0h 4h 16h
RhoB

Figure 1

B)


Control
FTI
RIE/neo
RIE/K-ras

C)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FTI
RIE/neo
RIE/K-ras

Figure 2

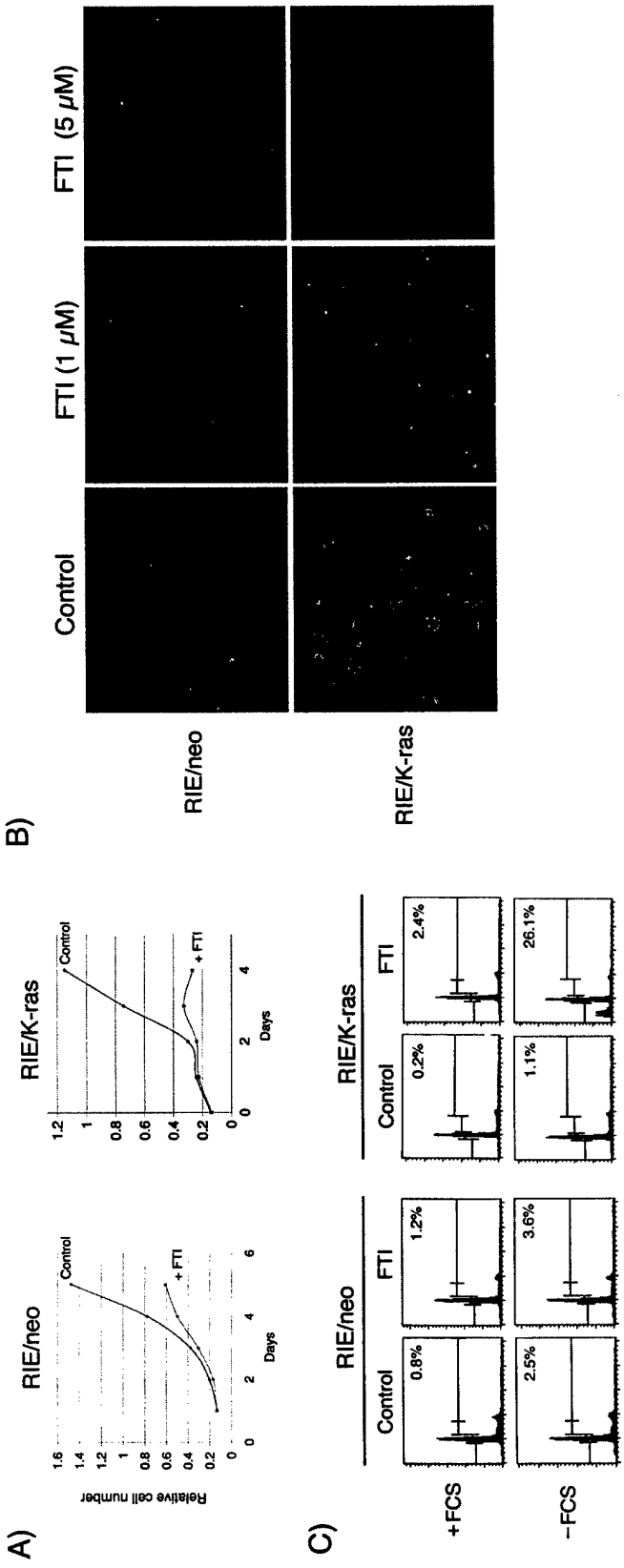

A)
RIE/neo
Control
+ FTI
Relative cell number
Days
RIE/K-ras
Control
+ FTI
Days
B)
Control
FTI (1 μM)
FTI (5 μM)
RIE/neo
RIE/K-ras
C)
RIE/neo
Control
FTI
0.8%
1.2%
2.5%
3.6%
RIE/K-ras
Control
FTI
0.2%
2.4%
1.1%
26.1%
+FCS
-FCS

Figure 3

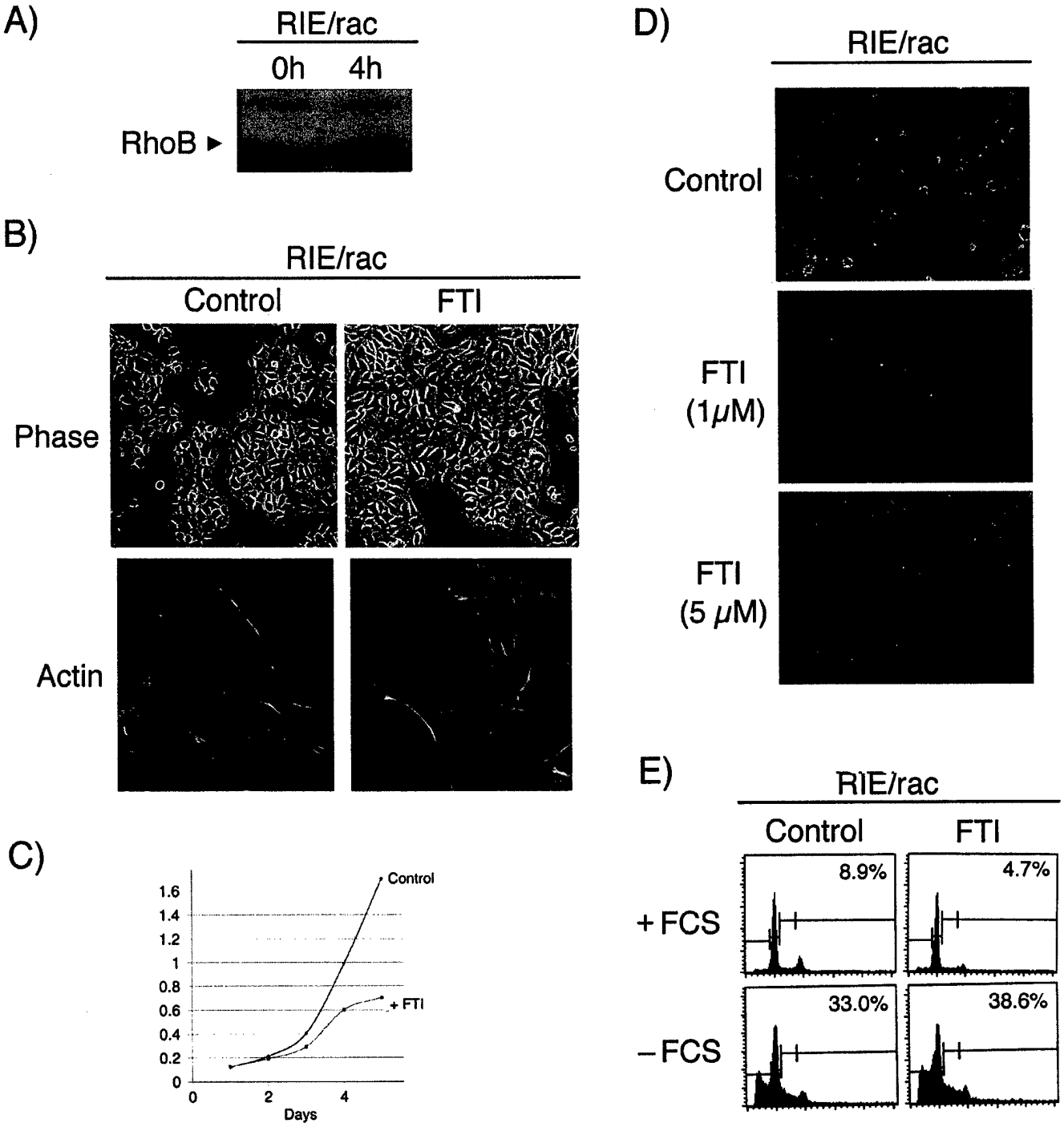

A)
RIE/rac
0h
4h
RhoB ▶
B)
RIE/rac
Control
FTI
Phase
Actin
C)
Control
+ FTI
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
Days
D)
RIE/rac
Control
FTI (1μM)
FTI (5 μM)
E)
RIE/rac
Control
FTI
+ FCS
8.9%
4.7%
– FCS
33.0%
38.6%

Figure 4

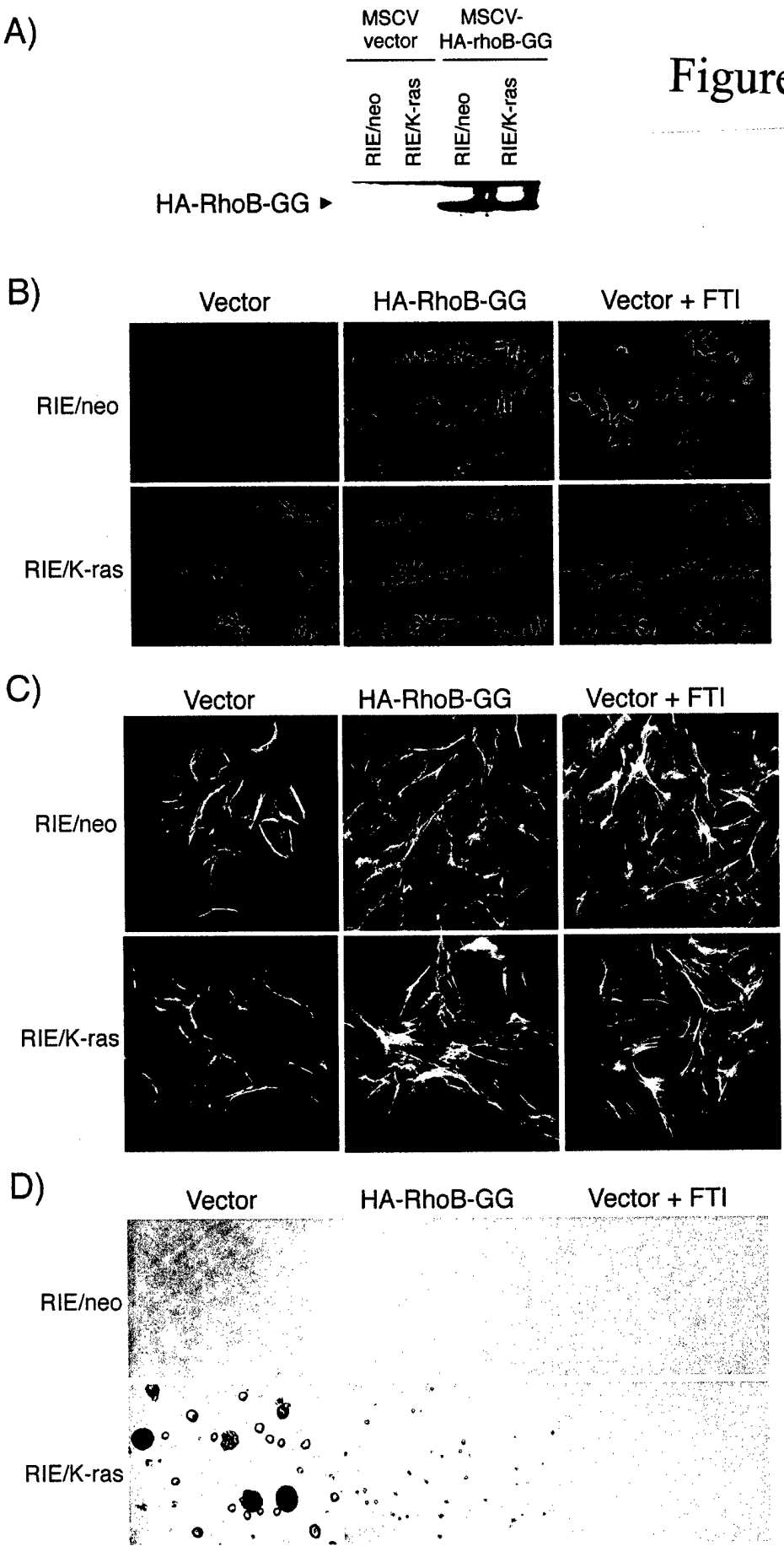

A)
MSCV vector
MSCV-HA-rhoB-GG
RIE/neo
RIE/K-ras
RIE/neo
RIE/K-ras
HA-RhoB-GG ▸
B)
Vector
HA-RhoB-GG
Vector + FTI
RIE/neo
RIE/K-ras
C)
Vector
HA-RhoB-GG
Vector + FTI
RIE/neo
RIE/K-ras
D)
Vector
HA-RhoB-GG
Vector + FTI
RIE/neo
RIE/K-ras

Figure 5

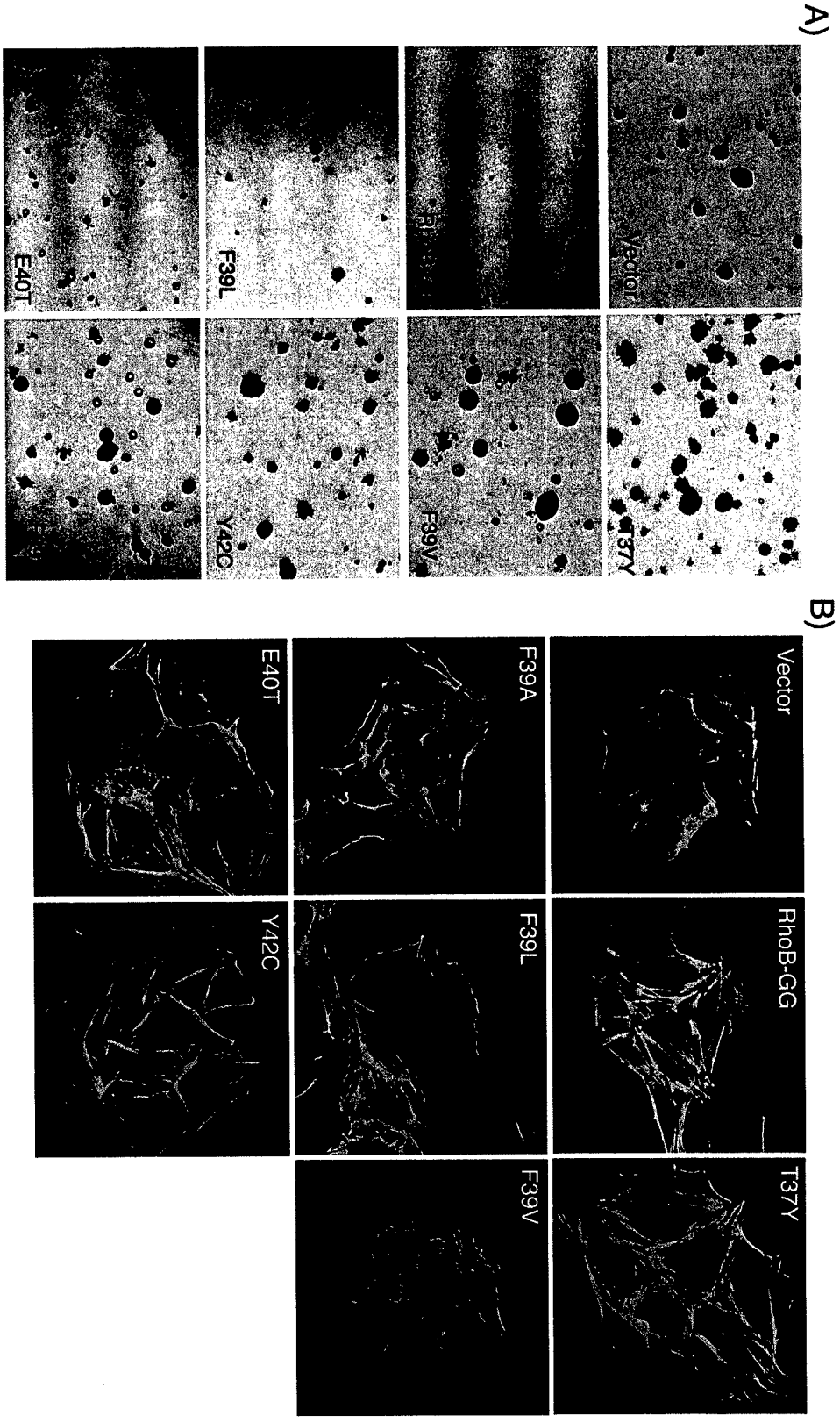

A)
Vector
T37Y
F39L
F39V
E40T
Y42C
B)
Vector
RhoB-GG
T37Y
F39A
F39L
F39V
E40T
Y42C

Figure 6

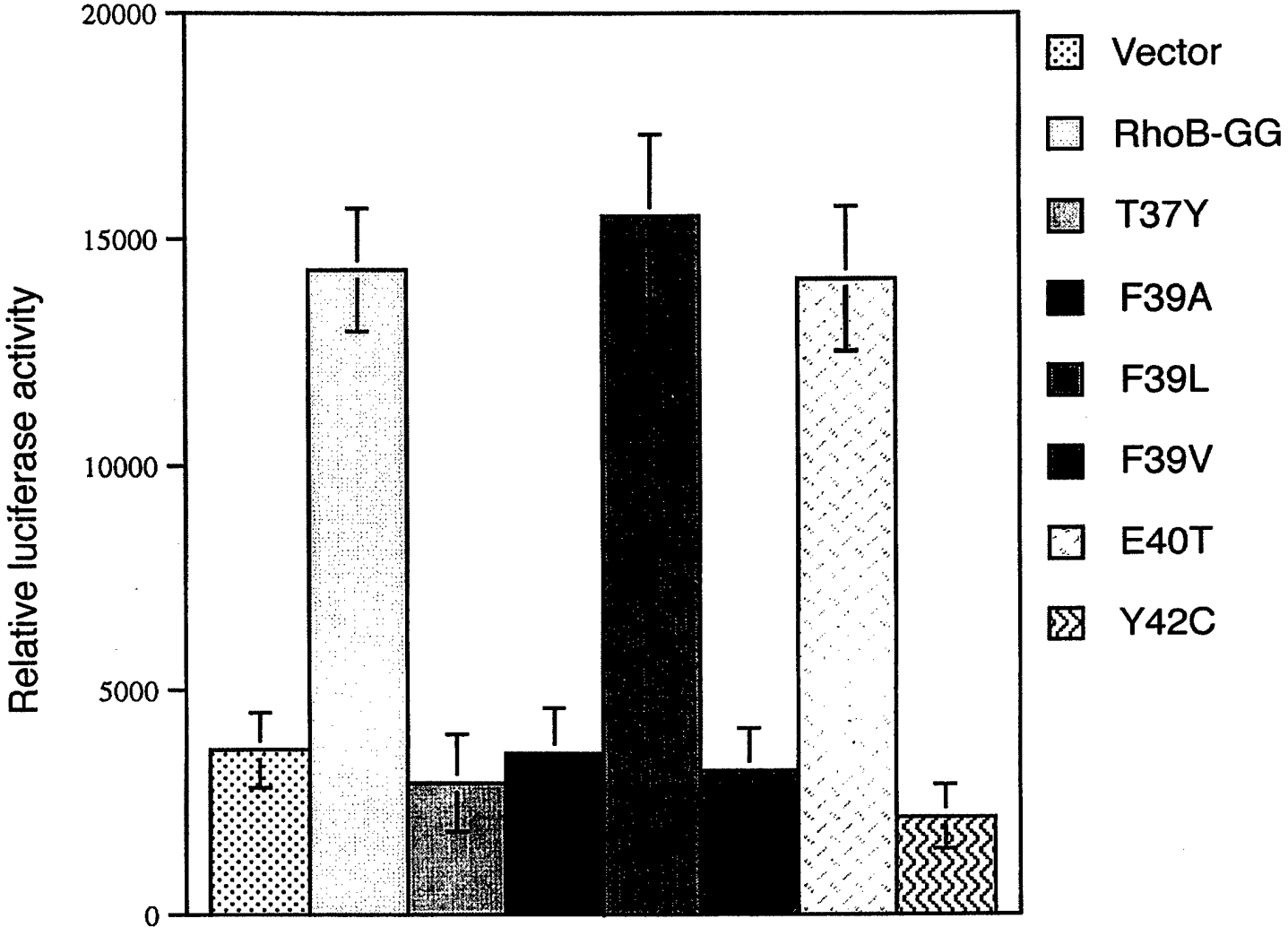
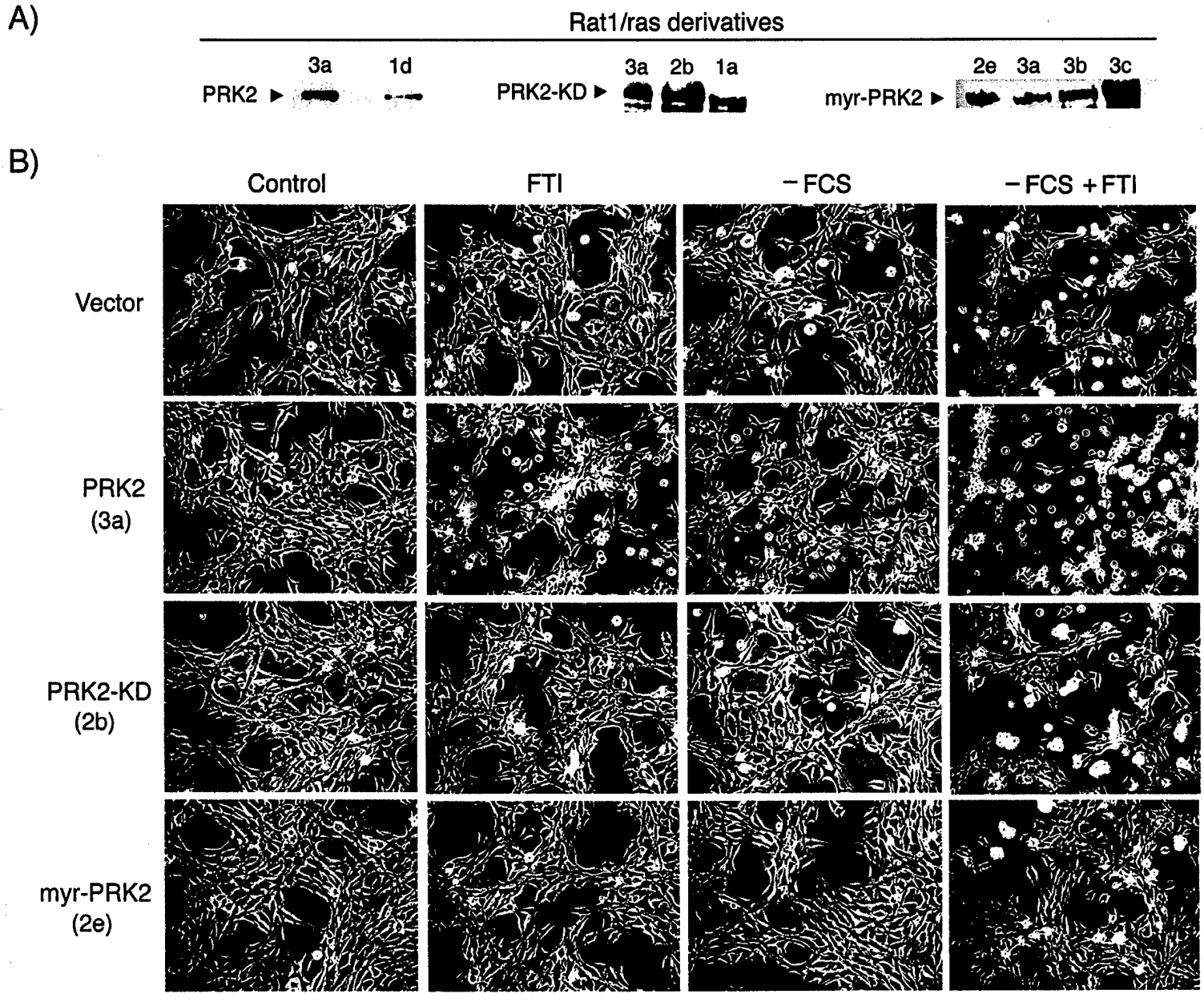

20000
15000
10000
5000
0
Relative luciferase activity
Vector
RhoB-GG
T37Y
F39A
F39L
F39V
E40T
Y42C

Figure 7


A)
Rat1/ras derivatives
PRK2
3a
1d
PRK2-KD
3a
2b
1a
myr-PRK2
2e
3a
3b
3c
B)
Control
FTI
−FCS
−FCS +FTI
Vector
PRK2
(3a)
PRK2-KD
(2b)
myr-PRK2
(2e)

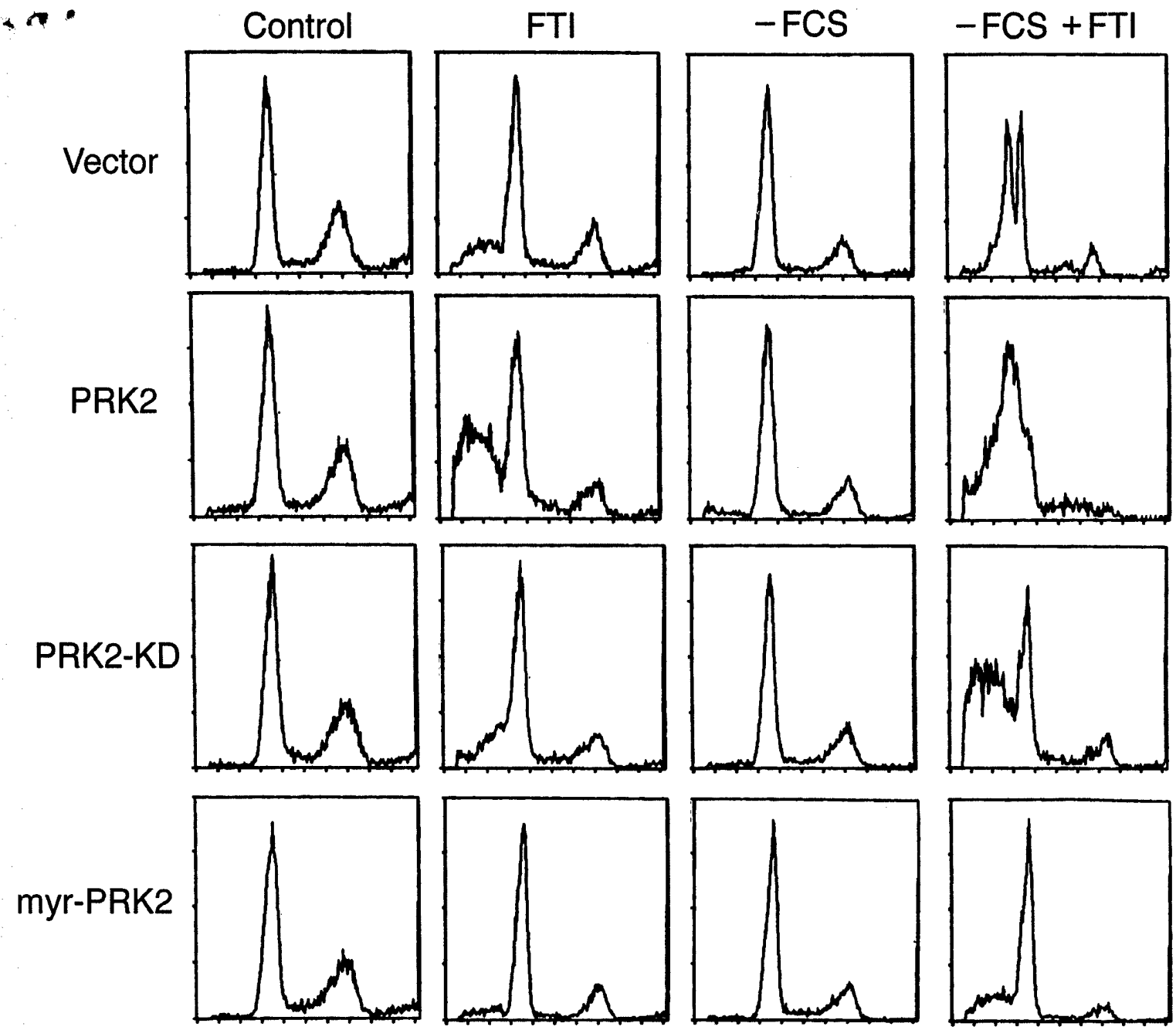


Figure 8